From gene to disorder in ADHD

Mapping mechanisms at different levels of complexity

Marieke Klein

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PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.H.J.M. van Krieken,
volgens besluit van het college van decanen
in het openbaar te verdedigen op vrijdag 1 februari 2019
om 10.30 uur precies

door

Marieke Klein
geboren op 29 juni 1988
te Leer (Duitsland)

ISBN: 978-94-6375-194-0
Cover design: Robbert de Vries, persoonlijkproefschrift.nl
Lay-out: Robbert de Vries, persoonlijkproefschrift.nl
Printing: Ridderprint BV, Ridderkerk, the Netherlands

Acknowledgements
This work was supported by funding provided by the Donders Institute and through a personal Vici grant from the Netherlands Organisation for Scientific Research (NWO grant 016-130- 669, to Barbara Franke). Part of this work was carried out on the Dutch national e-infrastructure with the support of SURF Foundation.

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DOCTORAL THESIS

to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. dr. J.H.J.M. van Krieken,
according to the decision of the Council of Deans
to be defended in public on Friday, February 1, 2019
at 10.30 hours

by

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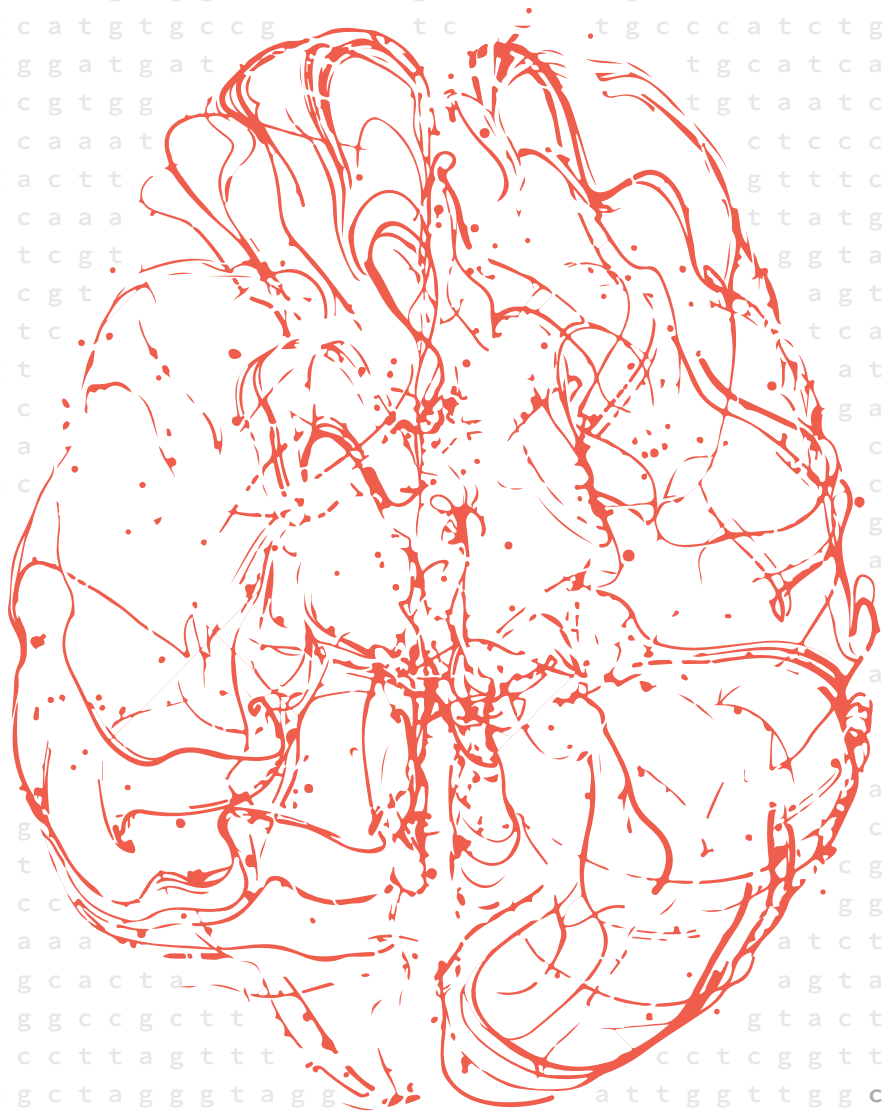

CHAPTER 1

Introduction

This thesis is aimed at gaining insight into the genetic underpinnings and underlying biological mechanisms of the highly heritable disorder Attention-Deficit/Hyperactivity Disorder (ADHD). The first part of this thesis focuses on the investigation of genetic risk factors for ADHD and related phenotypes employing established and novel molecular genetics approaches. The second part describes different strategies to map the pathways from gene to disorder at different levels of complexity and explores a diverse suite of model systems. In the following sections, a brief summary is presented about the clinical manifestation of ADHD, recent advances in the search for genetic risk factors associated with the disorder, and different methods for mapping the mechanisms underlying these risk genes, especially brain imaging genetics and the use of animal models. Subsequently, the aims of this thesis and an outline of the chapters are presented.

Attention-Deficit/Hyperactivity Disorder (ADHD)

ADHD is a common neurodevelopmental disorder (Faraone et al., 2015b). The world-wide prevalence has been estimated at 5% in children and at 2.5-4.9% in adults (Polanczyk and Rohde, 2007; Simon et al., 2009). ADHD is characterized by age-inappropriate levels of inattention and/or hyperactivity and impulsivity (Frances, 2000), but the clinical phenotype is heterogeneous (American Psychiatric Association, 2013; Frances, 2000) (**Box 1**). Approximately 55-75% of patients still carry the diagnosis in adulthood or remit only partially and display several impairments still in adulthood (Faraone et al., 2006). Severity level and presentation of ADHD symptoms can change during the lifespan; adults usually display less symptoms of hyperactivity and impulsivity (Buitelaar et al., 2011; Haavik et al., 2010).

ADHD is clinically diagnosed using structured interviews according to the Diagnostic and Statistical Manual of Mental Disorders (DSM; (American Psychiatric Association, 2013)) or the International Classification of Diseases (ICD; (World Health, 1992)). The current DSM-5 diagnostic criteria were published in 2013 and for a clinical diagnosis, symptoms must have emerged before the age of twelve years and have persisted for at least six months (**Box 1**). Generally, the symptoms must manifest "to a degree that is inconsistent with developmental level and that negatively impacts directly on social and academic/occupational activities" (American Psychiatric Association, 2013). For a clinical diagnosis of adult ADHD, the cutoff is at five symptoms which is based on literature (Kessler et al., 2010; Kooij et al., 2005) and scientific consensus.

Box 1. DSM-5 Diagnostic criteria for ADHD

A. A persistent pattern of inattention and/or hyperactivity that interferes with functioning or development, as characterized by (1) and/or (2):

1. Inattention: Six (or more) of the following symptoms have persisted for at least 6 months to a degree that is inconsistent with developmental level and that negatively impacts directly on social and academic/occupational activities:
Note: The symptoms are not solely a manifestation of oppositional behavior, defiance, hostility, or a failure to understand tasks or instructions. For older adolescents and adults (age 17 and older), at least five symptoms are required.

a. Often fails to give close attention to details or makes careless mistakes in schoolwork, at work, or during other activities (e.g., overlooks or misses details, work is inaccurate).
b. Often has difficulty sustaining attention in tasks or play activities (e.g., has difficulty remaining focused during lectures, conversations, or lengthy reading).
c. Often does not seem to listen when spoken to directly (e.g., mind seems elsewhere, even in the absence of any obvious distraction).
d. Often does not follow through on instructions and fails to finish schoolwork, chores, or duties in the workplace (e.g., starts tasks but quickly loses focus and is easily sidetracked).
e. Often has difficulty organizing tasks and activities (e.g., difficulty managing sequential tasks; difficulty keeping materials and belongings in order; messy, disorganized work; has poor time management; fails to meet deadlines).
f. Often avoids, dislikes, or is reluctant to engage in tasks that require sustained mental effort (e.g., schoolwork or homework; for older adolescents and adults, preparing reports, completing forms, reviewing lengthy papers).
g. Often loses things necessary for tasks or activities (e.g., school materials, pencils, books, tools, wallets, keys, paperwork, eyeglasses, mobile telephones).
h. Is often easily distracted by extraneous stimuli (for older adolescents and adults, may include unrelated thoughts).
i. Is often forgetful in daily activities (e.g., doing chores, running errands; for older adolescents and adults, returning calls, paying bills, keeping appointments).

2. Hyperactivity and impulsivity: Six or more of the following symptoms have persisted for at least 6 months to a degree that is inconsistent with developmental level and that negatively impacts directly on social and academic/occupational activities:
Note: The symptoms are not solely a manifestation of oppositional behavior, defiance, hostility, or a failure to understand tasks or instructions. For older adolescents and adults (age 17 and older), at least five symptoms are required.

a. Often fidgets with or taps hands or feet or squirms seat.
b. Often leaves seat in situations when remaining seated is expected (e.g., leaves his or her place in the classroom, in the office or other workplace, or in other situations that require remaining in place).
c. Often runs about or climbs in situations where it is inappropriate. (Note: In adolescents or adults, may be limited to feeling restless.)
d. Often unable to play or engage in leisure activities quietly.
e. Is often "on the go", acting as if "driven by a motor" (e.g., is unable to be or uncomfortable being still for extended time, as in restaurants, meetings; may be experiences by others as being restless or difficult to keep up with).
f. Often talks excessively.
g. Often blurts out an answer before a question has been completed (e.g., complements people's sentences; cannot wait for turn in conversation).
h. Often has difficulty waiting his or her turn (e.g., while waiting in line).
i. Often interrupts or intrudes others (e.g., butts into conversations, games, or activities; may start using other people's things without asking or receiving permission; for adolescents and adults, may intrude into or take over what others are doing).

Box 1. Continued.

In addition, the following conditions must be met:

B. Several inattentive or hyperactive-impulsive symptoms were present prior to age 12 years.

C. Several inattentive or hyperactive-impulsive symptoms are present in two or more settings (e.g., at home, school, or works; with friends or relatives; in other activities).

D. There is clear evidence that the symptoms interfere with, or reduce the quality of social, academic, or occupational functioning.

E. The symptoms do not occur exclusively during the course of schizophrenia or another psychotic disorder and are not better explained by another mental disorder (e.g., mood disorder, anxiety disorder, dissociative disorder, personality disorder, substance intoxication or withdrawal).

Specify whether:

Predominantly inattentive presentation: If Criterion A1 (inattention) is met but Criterion A2 (hyperactivity-impulsivity) is not met for the past 6 months.

Predominantly hyperactive-impulsive presentation: If Criterion A2 (hyperactivity-impulsivity) is met and Criterion A1 (inattention) is not met for the past 6 months.

Combined presentation: If both Criterion A1 (inattention) and Criterion A2 (hyperactivity-impulsivity) are met for the past 6 months.

Specify if:

In partial remission: When full criteria were previously met, fewer than the full criteria have been met for the past 6 months, and the symptoms still result in impairment in social, academic, or occupational functioning.

Specify current severity:

Mild: Few, if any symptoms in excess of those required to make the diagnosis are present, and symptoms results in no more than minor impairments in social or occupational functioning.

Moderate: Symptoms or functional impairments between "mild" and "severe" are present.

Severe: Many symptoms in excess of those required to make the diagnosis, or several symptoms that are particularly severe, are present, or the symptoms result in marked impairment in social or occupational functioning.

Changes in DSM-5 compared to DSM-IV-TR

- Age at diagnosis: For many years, the diagnostic criteria for ADHD stated that it was children who were diagnosed with the disorder. This meant for older adolescents and adults with impairing symptoms of the disorder that they could not officially be diagnosed with ADHD. The DSM-5 has changed this; older adolescents and adults can now be officially diagnosed with the disorder. The diagnostic criteria mention and give examples of how the disorder appears in older adolescents and adults.
- In diagnosing ADHD in adults, clinicians now can look back to middle childhood (age 12 years) when evaluating the onset of symptoms, but do not have to go back all the way to early childhood (age 7 years).
- In the previous edition, DSM-IV-TR, the three types of ADHD were referred to as "subtypes". This has changed; subtypes are now referred to as "presentations". Furthermore, a person can change "presentations" during lifetime.
- Severity is now specified as mild, moderate, or severe ADHD. This is based on the impairment.
- A person can now be diagnosed with both ADHD and autism spectrum disorder.
- In making the diagnosis, children still should have six or more symptoms of the disorder. In older teens and adults the DSM-5 states they should have at least five symptoms.

As apprarent from **Box 1**, the DSM-5 recognizes three presentations: predominantly inattentive, predominantly hyperactive-impulsive, and combined. These diverse presentations can changes over time (Nigg et al., 2010) and even within such presentations patients greatly differ in symptom profiles (Faraone et al., 2015a). Thus, ADHD patients show large variation in symptom profiles, impairments, neuropsychological alterations and their underlying causes (Sonuga-Barke and Taylor, 2015). Heterogeneity in the clinical presentation of ADHD is also apparent from a diverse spectrum of psychiatric comorbidities; both frequently seen in children (Biederman and Faraone, 2005; Gillberg et al., 2004; Lycett et al., 2015; Rappley, 2005; Reinhardt and Reinhardt, 2013) and in adults (McGough et al., 2005; Miller et al., 2007; Ollendick et al., 2008; Sobanski et al., 2007; Wilens et al., 2009). This indicates that psychiatric comorbidities are an important factor for impairment, severity, and persistence of ADHD symptoms (Biederman et al., 2011; Lara et al., 2009). The most frequent comorbidities seen in ADHD are substance use disorders, anxiety and mood disorders, oppositional, defiant and conduct disorder, and autism spectrum disorder (Anckarsater et al., 2006; Wilens et al., 2009). Additionally, compared to the general population, ADHD is a common comorbid disorder in children with intellectual disability (ID) (Maulik and Harbour, 2010; Vorstman and Ophoff, 2013), and the risk of ID increases with increasing severity of ADHD (Voigt et al., 2006). Studies of children with mild ID have identified co-morbid ADHD in 8-39% of the cases (Baker et al., 2010; Dekker and Koot, 2003; Emerson, 2003).

Genetic architecture of ADHD

While ADHD is diagnosed at the behavioral level, it is a neurobiological disorder that is most likely caused by a complex interplay between genetic and environmental risk factors. It has been hypothesized that these risk factors affect the structure and function of brain networks and thereby ultimately lead to ADHD symptoms, neurocognitive deficits, and various functional impairments (Faraone et al., 2015a). The etiology of ADHD is strongly influenced by genetic factors, as demonstrated by twin and adoption studies (Burt, 2009; Faraone and Mick, 2010; Kotte et al., 2013; Thapar et al., 2013). ADHD heritability estimates range between 70 and 90% (Faraone and Mick, 2010; Larsson et al., 2013). Despite this high heritability, gene identification has been challenging (Franke et al., 2009; Glahn et al., 2007). One reason for this is the complex, polygenic background of ADHD. This means that multiple genetic variants, each of them with small effects, contribute to the etiology of the disorder in most patients. Although a substantial part of the ADHD etiology is due to genetic factors, many environmental risk factors and potentially also gene-environment interactions are associated with an increased risk for the disorder (Banerjee et al., 2007; Han et al., 2015).
Many different molecular genetics studies have been undertaken in order to study the genetic background of ADHD. Basically, the different methods used in this can be divided

into hypothesis-driven and hypothesis-generating approaches. As ADHD has a high prevalence in the population, the search for genetic factors started with common genetic variants, which are relatively frequent in the general population (>1%) and generally have small effect sizes (Li et al., 2014; Neale et al., 2010b) (**Figure 1**).

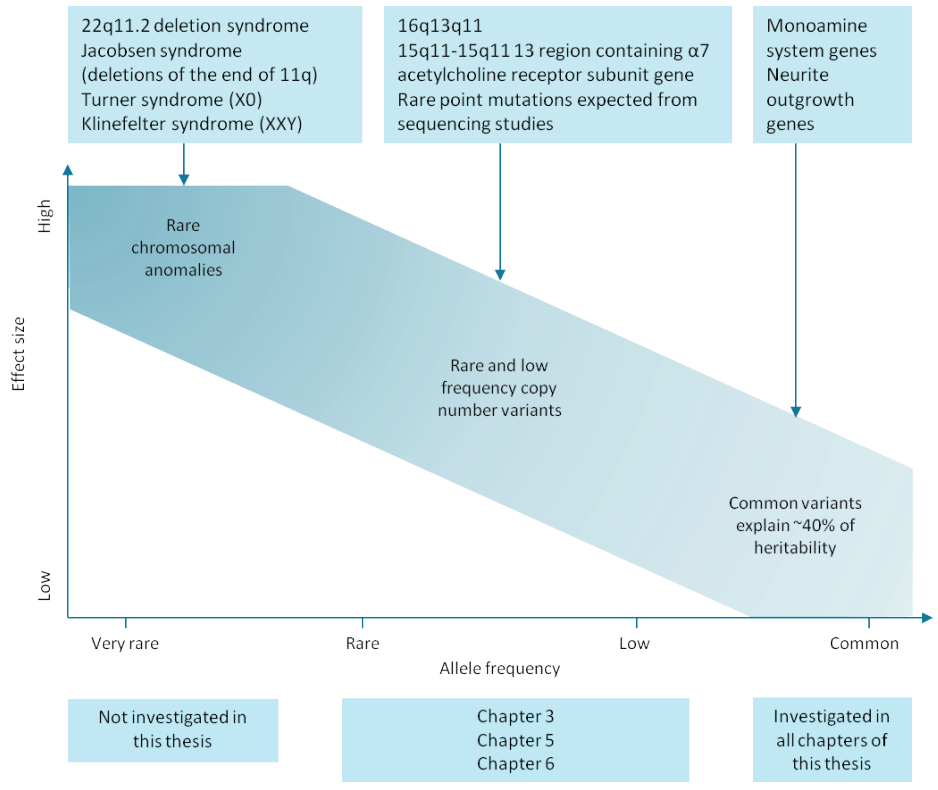

Figure 1. Relationship between allele frequency and effect size in ADHD. Common genetic variants explain approximately 40% of the heritability of ADHD, but compared with rarer variants, individual common variants have much smaller effects on the risk of the disorder. All chapters of this thesis investigated the role of common variants in ADHD. In addition, in chapter 3, 5, and 6, the contribution of rare and low frequency variants in ADHD was examined. SNPs = single nucleotide polymorphisms. The figure was adapted from (Faraone et al., 2015a).

Initially, only candidate gene-based association studies were performed. Although those were individually underpowered, meta-analyses identified several significant associations (Faraone et al., 2005; Gizer et al., 2009; Li et al., 2006) (reviewed elsewhere (Banaschewski et al., 2010; Faraone and Mick, 2010; Franke et al., 2012; Hawi et al., 2015; Li et al., 2014)), and most of the associated genes belong to monoaminergic neurotransmission systems and/or are neurite outgrowth genes.

Family-based approaches, i.e. genetic linkage analysis, provided a first possibility to carry out hypothesis-generating studies. In linkage studies, genetic variants across the entire genome are measured and tested for the odds of being shared between affected family members compared to the odds of not being shared. However, gene identification through linkage is limited (Banaschewski et al., 2010) to genetic variants with bigger effect sizes (**Figure 1**). A meta-analysis of seven linkage studies identified a locus on chromosome 16, encompassing the *CDH13* gene (Zhou et al., 2008), a gene that has also been found in the top-ranks of several genome-wide association studies (GWASs) (Lasky-Su et al., 2008; Neale et al., 2010a). Other successful linkage analysis results include the *LPHN3* gene on chromosome 4 (Arcos-Burgos et al., 2010), which was subsequently confirmed through association testing (Ribases et al., 2011).

The most frequently used hypothesis-generating approach is the GWAS, which investigates millions of common genetic markers (across the genome), so called single nucleotide polymorphisms (SNPs; **Figure 1**). Given the individual small effects of these genetic markers, those studies require large sample sizes (Holland et al., 2016; Smoller et al., 2018). With the recent success of international consortia, the first genome-wide significant hits for clinical ADHD are now becoming available (Demontis et al., 2017). Despite this success, the overall SNP-based heritability (h^2) has been estimated to be approximately 22% only, which means that most of the heritability is still unexplained. These estimates are lower than those derived from twin studies, in part because the SNP-based heritability only includes effects due to common variants.

Genetic studies of ADHD are usually carried out using the clinical diagnosis (i.e. case v. control) as primary outcome. This has been complemented and expanded by performing studies of quantitative measures of (childhood) ADHD symptoms in the population (e.g. EAGLE consortium (Middeldorp et al., 2016)). Both phenotypes, clinical ADHD and ADHD symptoms, show a strong genetic correlation, suggesting genetic overlap (Demontis et al., 2017). Most of the previously mentioned studies focused on childhood samples. A GWAS of persistent ADHD is currently in preparation (Ribases et al., 2017) and seems to show a very high genetic correlation with childhood ADHD, but the estimated SNP-based heritability is lower (h^2~16%) (Ribases et al., 2017). This suggests that also other types of genetic variation, and/or gene-gene and gene-environment interactions contribute to the persistence of ADHD. Recent studies show that ADHD is also genetically correlated with other psychiatric disorders (e.g., major depressive disorder), but also with more general anthropomorphic traits (e.g., body mass index) (Demontis et al., 2017). With methodological advances and novel statistical genetics approaches to assess genetic correlations by using GWAS summary statistics, the genetic overlap between disorders and traits is now extensively studied (Bulik-Sullivan et al., 2015a; Bulik-Sullivan et al., 2015b).

Following the promising findings in other psychiatric disorders (Cruceanu et al., 2013; Cukier et al., 2014; Kerner et al., 2013; Purcell et al., 2014), studies of rare genetic variants

have also been performed for ADHD. Several studies were successful in identifying genetic variants related to the disorder (Elia et al., 2010; Lesch et al., 2011; Ramos-Quiroga et al., 2014; Williams et al., 2012; Williams et al., 2010; Yang et al., 2013). Genome-wide analysis of (rare) copy number variants (CNVs) showed an enrichment of rare CNVs in patients with ADHD (Williams et al., 2010), and implicated the genes *CHRNA7* and *NPY* in ADHD etiology (Lesch et al., 2011; Williams et al., 2012), as well as genes encoding glutamate receptors (Akutagava-Martins et al., 2014; Elia et al., 2012), and regions on 15q11-15q13 (Valbonesi et al., 2015) and 16p13.11 (Williams et al., 2010). A recent re-analysis of available CNV data revealed 26 novel ADHD genes that were observed across a set of complementary approaches (Harich et al., submitted). Subsequent bioinformatic analysis implicated several biological processes affected in ADHD, including cell–cell junctions in the blood–brain barrier, transcriptional regulation, apoptosis, regulation of synaptic vesicles, and ion channel activity (Harich et al., submitted; Thapar et al., 2015). The picture emerging from those initial studies is that the contribution of rare genetic variants to ADHD etiology is highly heterogeneous, similar to the common variant contribution. Whole-exome sequencing (WES) and whole-genome sequencing (WGS) are now being increasingly used, allowing the identification of rare single nucleotide variants and small insertions/deletions contributing to ADHD etiology. As apparent from **Figure 1**, it is hypothesized that those rare variants have larger individual effect sizes. A first study indeed found enrichment of rare variants in a predefined set of 51 candidate genes in adult patients with persistent ADHD (Demontis et al., 2016). Additional candidate gene-based studies support the role of (*de novo*) rare variants in ADHD (Hawi et al., 2017; Kim et al., 2017).

Genetic factors associated with ADHD are widely spread across the genome, but they tend to be enriched within specific functional categories (Demontis et al., 2017; Thapar et al., 2015). Various approaches have been successful detecting ADHD-related gene clusters within various functional networks and biological processes that have been shown to be involved in the etiology of the disorder. GWAS association signals converged on the directed neurite outgrowth network, for example (Poelmans et al., 2011). Additionally, enrichment analyses showed that most significantly enriched function for ADHD-GWAS association signals were related to nervous system development, neuron projection morphogenesis, cell–cell communication, glutamatergic synapse/receptor signalling, and multicellular organismal development (Hawi et al., 2015; Yang et al., 2013). These findings were substantiated by a study that used two GWAS data sets to identify pathways associated with ADHD by applying six pathway analysis methods (Mooney et al., 2016).

Mapping mechanisms underlying ADHD risk genes

The effects of ADHD genetic risk factors on behavior are likely to be mediated through effects on cell biology and brain system development and functioning. This section emphasizes the importance of using complementary methods for the evaluation of mechanisms underlying ADHD risk genes. It also highlights two main approaches which are applied throughout the different chapters of this thesis.

Brain imaging genetics

ADHD is a disorder of the brain (Hoogman et al., 2017) and several aspects of brain development, structure, function, and connectivity have been found altered in ADHD (Cortese et al., 2013; Cortese et al., 2012; Greven et al., 2015; Mostert et al., 2016; Onnink et al., 2015; Shaw et al., 2007; Shaw et al., 2012; van Ewijk et al., 2012). The different neuroimaging techniques used and the main findings for ADHD are described in more detail in **chapter 8**. Briefly, structural magnetic resonance imaging (sMRI) has pointed to total brain volume and total grey matter reductions of up to 3-5% in patients with ADHD compared to controls (Castellanos et al., 2002; Greven et al., 2015; Valera et al., 2007). A recent meta-analysis revealed that, on average, patients with ADHD have a smaller intracranial volume (ICV) and smaller volumes of subcortical regions, such as the nucleus accumbens, amygdala, caudate nucleus, putamen, pallidum, thalamus, and of the hippocampus (Hoogman et al., 2017). Next to volumetric differences observed in grey matter, white matter structure has also been found to be altered in ADHD, leading to a potential disorganization of the brain's connectivity. Most consistently, studies reported white matter anomalies in the corpus callosum in childhood ADHD (van Ewijk et al., 2014) and adult ADHD (Dramsdahl et al., 2012; Onnink et al., 2015). A method to investigate potential changes in brain activity is functional magnetic resonance imaging (fMRI). Generally, dysregulation of structure and function of the fronto-subcortical-cerebellar pathways that control attention, response to reward, salience thresholds, inhibitory control, and motor behavior are among most consistently observed (del Campo et al., 2012; Gallo and Posner, 2016; Rommelse et al., 2011), and task-based functional MRI studies in ADHD have largely focused on these neurocognitive functions/domains (Cortese et al., 2012; Hart et al., 2012; Hart et al., 2013; Plichta and Scheres, 2014). Such brain phenotypes are often moderately to highly heritable. Twin studies showed that genetic effects varied regionally within the brain, with high heritability estimates (h^2) for frontal lobe volumes (ranging from 0.9 to 0.95) and for region-based cortical surface areas (ranging from 0.48 to 0.77), and moderate estimates for e.g. the hippocampus (h^2-range = 0.4–0.69) (Peper et al., 2007).

The brain phenotypes differing between patients and controls have been considered as intermediate phenotypes for the disorder, and investigating the genetic influences on these brain measures has been offered as a way for capturing underlying liability for ADHD

(Dresler et al., 2014; Durston, 2010; Wu et al., 2014). Such 'imaging genetics' approaches may provide more insight into the underlying biological mechanisms of genetic risk factors in ADHD. The main findings are reviewed in **chapter 7** and **chapter 8**. Basically, most studies focused on genotypic effects of single genetic variants of dopamine-related genes, such as *DAT1* and *DRD4*. Additionally, the majority of studies investigated the genotypic effects on structural MRI measures. So far, the reports have been inconsistent, making their results inconclusive. Next to the limited samples sizes, the current literature suffers from lack of homogeneity/comparability of study designs and analysis methodologies.

Animal models

Imaging genetics analyses of the human brain provide information on the effect of ADHD risk genes/variants on *in vivo* brain structure, activity, and connectivity, but other, more fine-grained levels of investigation are needed to understand the underlying biological mechanisms. In this thesis, small animal models, such as mice and *Drosophila melanogaster*, are used in order to shed light on the mechanisms underlying the effects of ADHD genes on behavior (and a proxy for disease) at additional levels of complexity.

The main advantages of using animal models are that they can be genetically modified to enable determination of causality, they have a natural complexity of the nervous system, and allow a tight control of environmental influences, such as diet and drug delivery (Lange et al., 2012; van der Voet et al., 2016).

In ADHD research, most knock-out and transgenic mouse models thus far have targeted dopaminergic genes. These mutant models provide an excellent opportunity to evaluate the contribution of dopamine-related processes to (ADHD) brain pathophysiology, to analyze the neuronal circuits and molecular mechanisms involved in the action of ADHD medication, and to test novel treatments for ADHD (for review see (Leo and Gainetdinov, 2013)). Early mouse studies were used to validate the involvement of candidate genes from association studies in ADHD, e.g. by showing ADHD-like behavioral phenotypes in *Dat1* knock-out mice and the *Snap25*-mutated coloboma (Giros et al., 1996; Heyser et al., 1995).

The main advantages of the fruit fly (*Drosophila melanogaster*) compared to rodent models are that this model is relatively inexpensive, has a wide range of genomic tools readily available, and is highly suitable for fast, high-throughput studies of (candidate) genes. The combination of those characteristics with the availability of valid and quantifiable phenotypic readouts for ADHD-relevant traits makes this animal model a potent addition to non-invasive studies in humans. In the fruit fly, manipulation of the orthologues of the dopamine-related genes *DAT1* and *LPHN3* caused characteristic darkness-dependent increased locomotor activity, an ADHD-like behavior (van der Voet et al., 2016). Also in these models, ADHD medication was able to reverse the behavioral phenotype (van der Voet et al., 2016).

Aim and structure of this thesis

The overall aim of the studies described in this thesis was to improve our understanding of the genetic underpinnings of ADHD and to map the biological pathways and mechanisms from gene to disorder, by using brain imaging genetics approaches and animal model systems.

The thesis is divided into two parts, according to two main research questions:

- Part 1 "**Approaches for gene identification"**, tries to answer the question which molecular genetics mechanisms are underlying ADHD etiology employing a diverse set of established and innovative approaches.
- Part 2 "**Mapping of mechanisms from gene to disorder"**, describes different approaches, such as the use of brain imaging genetics and animal models, to characterize and understand functional role of the genetic effects involved in ADHD.

Part 1. Approaches for gene identification (Chapter 2-6)

As the search for genetic risk factors for ADHD has proven to be difficult, the aim of the first part of this thesis was to work on established and novel study designs that could help with identifying or rejecting involvement of genetic mechanisms underlying ADHD risk. In order to capture the entire spectrum of genetic variations, both common genetic variants and rare (private) mutations were investigated. Instead of focusing on the clinical diagnosis only, we also used quantitative ADHD scores as research outcomes and employed a lifespan view on the disorder. Depending on the specific research question we asked, versatile analytical methodologies were applied, ranging from the hypothesis-driven, meta-analytic investigation of single genetic variants to genome-wide, hypothesis-generating work.

In **chapter 2**, we studied the association of the childhood ADHD-associated variable number tandem repeat (VNTR) polymorphism upstream of gene encoding the dopamine D5 receptor, *DRD5*, with persistent ADHD. We compiled data from six sites of the International Multicentre persistent ADHD CollaboraTion (IMpACT) and investigated the largest sample to date. We tested the association of common *DRD5* alleles with categorical ADHD status (case/control) and with inattentive and hyperactive/impulsive symptom counts. In **chapter 3**, we also investigated common genetic variants, but now applying a hypothesis-free approach. Here, we report results of a genome-wide association meta-analysis on self-reported adult ADHD symptoms of nine adult population-based and case-only cohorts with subsequent rare variant analysis, and validation of findings in childhood population samples and mouse models. In **chapter 4**, we aimed to identify novel ADHD genes by studying the genetic overlap between ID and ADHD. This was motivated by the fact that ADHD and ID often occur comorbid and that shared molecular genetic influences (mainly based on rare CNVs) have been suggested. We thus investigated, whether genes known to carry rare

mutations in ID contribute to ADHD risk through common variants. Validation and functional characterization of two candidates was performed using *Drosophila melanogaster*. In the next two chapters, we focused on less frequent genetic variation. In **chapter 5**, we used a multi-step approach to identify and validate a novel ADHD risk gene. In a single family, severely affected by ADHD and comorbid disorders, we applied microarray analysis to detect rare structural genetic variants co-segregating with ADHD. One gene was taken forward for functional validation using *Drosophila melanogaster* as a biological model system. In **chapter 6**, we explored, whether combining linkage analysis and WES in large multi-generational pedigrees with association testing in an independent adult ADHD exome-chip study is a viable approach to gene-finding in (persistent) ADHD.

Part 2. Mapping of mechanisms from gene to disorder (Chapter 7-10)
In addition to the mechanistic work that made part of **chapters 3** and **4**, the second part of the thesis focuses on diverse approaches that can help to shed light on the different pathways involved in ADHD etiology. In **chapter 7**, we reviewed the imaging genetics literature on three neurodevelopmental disorders, which often co-occur (ID, ASD, and ADHD), attempting to understand the genetic mechanisms underlying individual disorders and their clinical overlap. For ADHD and ASD, genes showing replicated associations through common genetic variants with either disorders were selected. For ID, which is mainly caused by rare variants, we included genes for relatively frequent forms of ID occurring comorbid with ADHD or ASD. We reviewed case-control studies and studies of risk variants in healthy individuals. In **chapter 8**, we performed a systematic review of brain imaging genetics studies involving 62 ADHD candidate genes in childhood and adult ADHD cohorts. Beyond reviewing brain imaging genetics studies, we also discussed the need for complementary approaches at multiple levels of biological complexity and emphasized the importance of combining and integrating findings across levels for a better understanding of biological pathways from gene to disease. In **chapter 9**, we aimed at replicating the association of *GIT1* with ADHD and investigated its role in cognitive and brain phenotypes. Furthermore, functionality of a single genetic variant as an expression quantitative trait locus (eQTL) for *GIT1* was assessed in human blood samples. Using *Drosophila melanogaster* as a biological model system, we manipulated Git expression according to the outcome of the blood gene expression analysis and studied the effect of *Git* knockdown on neuronal morphology and locomotor activity. Our findings suggested that genetic risk for ADHD is mediated by alteration in structure and function of diverse brain networks. In **chapter 10**, we tested one aspect of this hypothesis. We investigated the genetic overlap between ADHD and (subcortical) brain volumes, both at the level of common variant genetic architecture as well as on the level of single variants. For this, we used the largest publicly available genome-wide genetic association data sets on ADHD risk and (subcortical) brain volumes.

Study cohorts and consortia

IMpACT

The International Multicentre persistent ADHD CollaboraTion (IMpACT; www.impactadhdgenomics.com) is a consortium of clinical and basic researchers from several European countries (The Netherlands, Germany, Spain, Norway, The United Kingdom, Sweden), the United States of America, and Brazil. The mission of IMpACT is to perform and promote high quality research in ADHD across the lifespan (Franke and Reif, 2013). This research is aimed at identifying novel genetic variants for adult ADHD and at understanding the mechanisms underlying the effect of these genetic variants on disease risk. IMpACT members share data on their samples as well as biological material, which has resulted in the formation of the largest database for persistent ADHD research. In this thesis, different types of data from IMpACT were used (**Table 1**).

PGC ADHD Working Group

The ADHD Workgroup was formed in 1998. This workgroup focuses on the study of ADHD and associated features in children and adults. In 2007, it joined the Psychiatric Genomics Consortium (PGC; http://www.med.unc.edu/pgc/pgc-workgroups). Current goals are to increase the number of ADHD samples with genome-wide association data and to extend the work into genome/exome sequencing. In this thesis, GWAS meta-analysis summary statistics of the PGC ADHD samples, sometimes in combination with data from iPSYCH, was used (**Table 1**).

iPSYCH

The Lundbeck Foundation Initiative for Integrative Psychiatric Research (iPSYCH; http://ipsych.au.dk/; (Pedersen et al., 2017)) ADHD sample is a nationwide population-based case-cohort sample selected from a baseline birth cohort comprising all singletons born in Denmark between May 1, 1981, and December 31, 2005, who were residents in Denmark on their first birthday and who have a known mother. Cases were diagnosed by psychiatrists at psychiatric hospitals (in- or out-patient clinics) according to ICD10 (F90.0), identified using the Danish Psychiatric Central Research Register (DPCRR). Controls were randomly selected from the same nationwide birth cohort and not diagnosed with ADHD (F90.0) or moderate-severe mental retardation (F71-F79). In this thesis, GWAS meta-analysis summary statistics of the iPSYCH ADHD sample was either used individually, or in combination with the PGC ADHD data (**Table 1**).

SAGA

The Study of ADHD trait Genetics in Adults (SAGA) consortium aims to identify ADHD risk genes by investigating the association between common genetic variants and adult ADHD symptom scores. The SAGA consortium carried out a GWAS meta-analysis in nine adult population-based and case-only cohorts of European Caucasian origin (age 18 years or

older) for whom adult ADHD symptom scores were available: six population-based cohorts, including one genetic isolate, two clinical ADHD samples, and one clinical cohort ascertained for depressive and anxiety disorders (**Table 1**).

EAGLE

The EArly Genetics and Lifecourse Epidemiology Consortium (EAGLE) is a consortium of pregnancy and birth cohorts that aims to collaborate to investigate the genetic basis of phenotypes related to physical and mental health from antenatal and early life to adolescence (https://www.wikigenes.org/e/art/e/348.html). In this thesis, summary statistic data of the EAGLE GWAS meta-analysis on childhood ADHD symptoms was used (Middeldorp et al., 2016) (**Table 1**).

BIG

The Brain Imaging Genetics (BIG) study was set up in 2007 by the Human Genetics department of the Radboud university medical center and the Donders Centre for Cognitive Neuroimaging of the Radboud University (www.cognomics.nl/big). In 2010, the Max Planck Institute for Psycholinguistics in Nijmegen also joined. BIG aims to identify the genetic factors influencing behavior, cognition, and brain structure and function in health and disease in individuals from the general population. The BIG database consists of brain imaging data, genetic data, and results of cognitive tasks and questionnaires of healthy adults subjects recruited in the Nijmegen area (**Table 1**).

ENIGMA

The Enhancing NeuroImaging Genetics through Meta Analysis (ENIGMA) network brings together researchers in imaging genomics to investigate brain structure, function, and disease, based on brain imaging and genetic data (http://enigma.ini.usc.edu/). ENIGMA combines numerous studies, including studies with a case-control design, which performed neuroimaging in a range of neuropsychiatric or neurodegenerative diseases, as well as studies of healthy/general populations. In this thesis, summary statistic data of the ENIGMA GWAS meta-analysis on subcortical volumes and intracranial volume was used (Adams et al., 2016; Hibar et al., 2017; Hibar et al., 2015) (**Table 1**).

CHARGE

The Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE, http://www.chargeconsortium.com/) (Psaty et al., 2009) consortium is a collaboration of predominantly population-based cohort studies that investigate the genetic and molecular underpinnings of age-related complex diseases, including those of the brain. In this thesis, summary statistic data of the CHARGE GWAS meta-analysis on hippocampal volume and intracranial volume was used (Adams et al., 2016; Hibar et al., 2017) (**Table 1**).

Table 1. Overview of different cohorts and data sets used in the thesis. Sample size and genetic data refer to the data that was used in this thesis.

Study name	Trait/phenotype of interest	Sample size	Genetic data	Website	Reference	Chapter
IMpACT NL	Persistent ADHD, ADHD symptoms, brain volume, neuropsychological variables	≥ 255	KASP genotyping, *DRD5* VNTR genotyping, genome-wide genotyping	www.impactadhdgenomics.com	(Klein et al., 2016; Klein et al., 2015)	2, 3, 9
IMpACT GER families	Persistent ADHD	≥ 70	WES, genome-wide, single variant genotyping			6
IMpACT international	Persistent ADHD, ADHD symptoms	≥ 6,979	Exome-chip, *DRD5* VNTR genotyping		(Klein et al., 2016; Zayats et al., 2016)	2,6
PGC ADHD	Childhood ADHD	19,210	GWAS summary statistics	http://www.med.unc.edu/pgc/results-and-downloads	(Holmans, 2013)	4, 9
iPSYCH ADHD	ADHD	37,076	GWAS summary statistics	http://ipsych.au.dk/	(Pedersen et al., 2017)	4
PGC+iPSYCH ADHD	ADHD	55,374	GWAS summary statistics	http://www.med.unc.edu/pgc/results-and-downloads	(Demontis et al., 2017)	5, 4, 3, 10
SAGA	Self-reported adult ADHD symptoms	14,689	GWAS summary statistics			3
EAGLE	Childhood ADHD symptoms	14,776	GWAS summary statistics	https://www.wikigenes.org/e/art/e/348.html	(Middeldorp et al., 2016)	3
BIG	sMRI segmentations	1,300	Genome-wide imputed dosages and genotypes	www.cognomics.nl/big	(Guadalupe et al., 2014)	9
ENIGMA	sMRI segmentations	≥ 11,221	GWAS summary statistics	http://enigma.ini.usc.edu/	(Adams et al., 2016; Hibar et al., 2017; Hibar et al., 2015; Thompson et al., 2014)	10
CHARGE	sMRI segmentations	≥ 12,803	GWAS summary statistics	http://www.chargeconsortium.com/	(Adams et al., 2016; Hibar et al., 2017; Psaty et al., 2009)	10

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PART 1

Approaches for gene identification

CHAPTER 2

Meta-analysis of the *DRD5* VNTR in persistent ADHD

Published as:

M. Klein, S. Berger, M. Hoogman, J. Dammers, R. Makkinje, A. J. Heister, T. E. Galesloot, L. A. Kiemeney, H. Weber, S. Kittel-Schneider, K. P. Lesch, A. Reif, M. Ribases, J. A. Ramos-Quiroga, B. Cormand, T. Zayats, T. A. Hegvik, K. K. Jacobsen, S. Johansson, J. Haavik, N. R. Mota, C. H. Bau, E. H. Grevet, A. Doyle, S. V. Faraone, A. Arias-Vasquez, and B. Franke (2016). "Meta-analysis of the *DRD5* VNTR in persistent ADHD." Eur Neuropsychopharmacol **26**(9): 1527-1532.

Abstract

Attention-Deficit/Hyperactivity Disorder (ADHD) is a common neuropsychiatric disorder with a complex genetic background. *DRD5*, the gene encoding the dopamine receptor D5, was recently confirmed as a candidate gene for ADHD in children through meta-analysis. In this study, we aimed at studying the association of the ADHD-associated variable number tandem repeat (VNTR) polymorphism upstream of *DRD5* with adult ADHD. We compiled data from six sites of the International Multicentre persistent ADHD CollaboraTion (IMpACT) and reached N=6,979 (3,344 cases and 3,635 healthy participants), the largest sample investigated so far. We tested the association of the common *DRD5* alleles with categorically defined ADHD and with inattentive and hyperactive/impulsive symptom counts. Our findings provide evidence that none of the common *DRD5* alleles are associated with ADHD risk or ADHD symptom counts in adults.

Keywords: *DRD5;* Attention-Deficit/Hyperactivity Disorder; candidate gene; meta-analysis

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a multifactorial neurodevelopmental disorder with an onset before the age of 12 years. It is characterized by hyperactivity, restlessness, impulsivity, and/or inattention (American Psychiatric Association, 2013). In a considerable number of patients, the disorder persists into adulthood, with a worldwide prevalence of ADHD of 1-4% in adults (Simon et al., 2009). Although ADHD is highly heritable in both children and adults (Faraone et al., 2005), only a few genetic risk factors have consistently been associated with ADHD risk, and most variants investigated are related to genes in the dopaminergic and serotonergic neurotransmission systems (Gizer et al., 2009). The dopamine receptor D5 (*DRD5*) gene is one of these; a variable number of tandem repeat polymorphism (VNTR) with a highly polymorphic dinucleotide repeat located 18.5 kb upstream in the 5′ region of the gene produces multiple alleles, of which two have been associated with ADHD. The association was first described in 1999 (Daly et al., 1999); since then, several studies investigated the effect of the *DRD5* VNTR on ADHD. A previous meta-analysis, including 12 studies (approximately 2,350 childhood cases and their parents), showed that the 148-bp allele is associated with increased ADHD risk, and that the 136-bp confers reduced ADHD risk (n=4 studies) (Wu et al., 2012). Mixed results for the role of the *DRD5* VNTR in persistent ADHD have been reported previously (Carpentier et al., 2013; Johansson et al., 2008; Langley et al., 2009; Squassina et al., 2008). To clarify the association of the *DRD5* VNTR with ADHD in adulthood, we performed a meta-analysis of case-control cohorts from six sites (Brazil, Germany, The Netherlands, Norway, Spain, and USA; n=6,979) of the International Multicentre persistent ADHD CollaboraTion (IMpACT, (Franke and Reif, 2013)). We tested the association of the DRD5 148-bp and 136-bp alleles with categorically defined ADHD and also with inattentive and hyperactive/impulsive symptom counts, separately. Subsequently, we performed exploratory analyses investigating the effects of ten additional, frequent DRD5 alleles.

Experimental Procedures

Subjects

Within the IMpACT consortium, all patients were evaluated by experienced psychiatrists and diagnosed with persistent ADHD according to DSM-IV (Diagnostic and Statistical Manual for Mental Disorders) criteria. Ratings of ADHD symptom counts were retrieved from clinical interviews, except for the Dutch and Norwegian cohorts, where symptom counts were derived from self-report questionnaires (see (Franke et al., 2010) for detailed information on diagnostic assessments). Participants were invited to provide whole blood or saliva samples for genotyping. Studies were approved by ethics committees of the participating

institutions, and written informed consent was obtained from all patients and controls prior to the study.

Genetic data

Genotyping of the *DRD5* (upstream VNTR) was performed in all six above-mentioned IMpACT cohorts by Fragment Length Analysis. The PCR reaction was performed on 30 ng genomic DNA using 0.33 µM 5'-fluorescently labeled (FAM, VIC, PET, or NED) forward primer (5'-GCTCATGAGAAGAATGGAGTG-3') and reverse primer with PIG tail (5'-CGTGTATGATCCCTGCAG-3') and 1x AmpliTaq Gold® 360 Master Mix (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). The cycling conditions for the polymerase chain reaction initiated with 10 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at the optimized annealing temperature (58°C), and 1 min at 72°C, then followed by a final step of 7 min at 72°C. The product of the amplification was diluted 1:30 in H2O. 1 ul of this dilution, together with 9.7 µl formamide and 0.3 µl Genescan-600 LIZ Size Standard (Applied Biosystems) was analyzed on an automated capillary sequencer (ABI3730 genetic analyzer, Applied Biosystems) according to the protocol of the manufacturer. To determine the length of the alleles, the results were analyzed with Genemapper software version 4.0 (Applied Biosystems). Output of the genotyping was the actual length in base pairs per allele, which was re-coded into three categories, i.e. homozygous of the reference allele, heterozygous, or homozygous for the non-reference allele.

The frequency distribution of the *DRD5* alleles (with a frequency >1%) across the different cohorts is shown in **Figure 1**. A total of 3,344 adult ADHD patients (44.4% female, average age = 34.9 years [range 18 – 75]) and 3,635 healthy individuals (51.7% female, average age = 39.1 years [range 18 – 92]) were available for the study. In all cohorts, allele frequencies were in Hardy-Weinberg equilibrium (HWE, P>0.05); the smallest cohort (USA, n=147) showed non-significant deviation from HWE (P=0.021, 12 *DRD5* alleles tested), which might be due to sampling variance in the underpowered cohort.

Statistical analyses

Output of the genotyping was the actual length in base pairs per allele, which was re-coded into three categories, i.e. homozygous of the reference allele, heterozygous, or homozygous for the non-reference allele. To prevent biases due to ethnic discrepancies, calculations were performed in each sample separately; subsequently, samples were subjected to meta-analysis (see below). A trend test was used to evaluate the ADHD risk conferred by carrying one of the *DRD5* allelic variants using logistic regression (additive model) for each individual IMpACT cohort. Analyses of symptom counts were performed separately for inattentive and hyperactive/impulsive symptoms in cases only. For this analysis, symptom count distributions were normalized and standardized using the Blom transformation. Impact of genotypes on inattentive and hyperactive/impulsive symptom scores was determined with

linear regression using genotype dosages (additive model) as independent and the trait of interest as dependent variable. Age and gender were included as covariates in all analyses. Twelve alleles of the *DRD5* VNTR were selected for analysis (**Figure 1**) and all data analyses were performed separately for each *DRD5* allele using the Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM Corp. Released 2011, IBM SPSS Statistics for Windows, Version 20.0 Armonk, NY: IBM Corp.).

All six cohorts were used to run a fixed effects meta-analysis model using the "rma" command implemented in the R package "metafor" v1.9-4 (Viechtbauer, 2010). As a measure for effect size either the odds ratios (ORs) or betas were calculated. Q-statistic and I2-metric were used to test and discard heterogeneity. When no heterogeneity was present, the pooled OR/beta was estimated using fixed effects model. The results of the association tests are indicated as pooled ORs/betas with the corresponding 95% confidence intervals (CIs) of the allele-induced risk of persistent ADHD or ADHD symptom counts.

Power analysis

Power analysis using the Genetic Power Calculator (GPC) (Purcell et al., 2003) and previously reported effect estimates showed that we had 64.1% power to detect effect sizes similar to the ones reported previously (OR≥1.26 for the 148-bp allele), 80% power to detect OR>1.4, and 100% power for effects of OR≤0.58 (for the 136-bp allele) (Wu et al., 2012). Also, our study had 90.1% power to detect associations explaining ≥0.5% of variance in ADHD symptom counts.

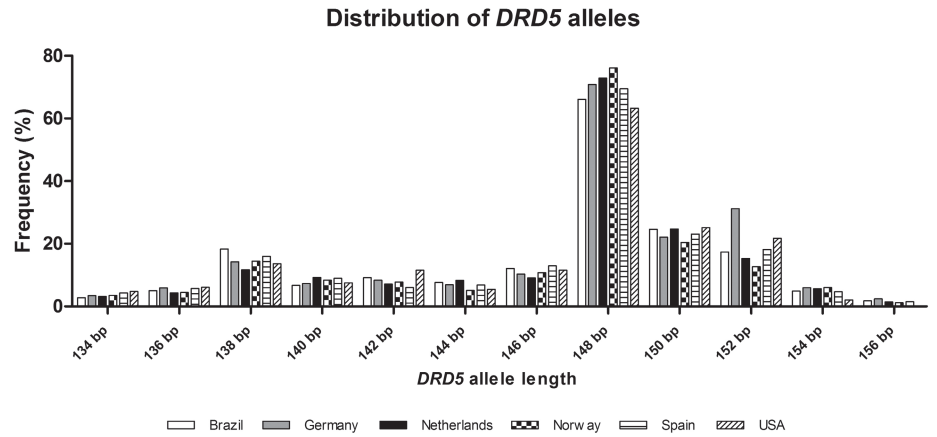

Figure 1: Distribution of *DRD5* VNTR alleles across all IMpACT cohorts included in the study. Only alleles with a minor allele frequency (MAF) >1% are shown.

Results

In total, 12 alleles of the *DRD5* VNTR were selected for analysis (**Figure 1**). Neither the 148-bp childhood ADHD risk allele nor the 136-bp childhood ADHD protective allele were associated with ADHD risk in adults (OR=0.97, 95% CI 0.90–1.04, P=0.425 and OR=0.92, 95% CI 0.73–1.15, P=0.464, respectively; **Figure 2A**). The same was found in the analysis of the separate ADHD symptom domains (N≥2,415; P>0.05, **Figure 2B,C**). An exploratory analysis investigating the ten additional *DRD5* alleles did not reveal any significant association with ADHD risk or ADHD symptom counts either (best p-value was P=0.07 for the 146-bp allele and ADHD risk). Overall results did not change using a random-effects model.

Discussion

While evidence for an association of *DRD5* VNTR alleles with ADHD in children had been strengthened by meta-analysis (Wu et al., 2012), we did not find similar effects in the current meta-analysis of persistent ADHD in adults. These differences are consistent with twin data that suggest the risk alleles contributing to ADHD symptoms partly differ by age (Chang et al., 2013; Pingault et al., 2015). Differential association in childhood and adulthood has also been reported for the dopamine transporter gene (DAT1/SLC6A3) (Franke et al., 2010). The potential age-dependent effect of *DRD5* on ADHD could be further investigated in longitudinal study designs. However, we cannot entirely exclude the chance of a false negative finding for the 148-bp allele, for which we had limited power of 64.1%. Notably, earlier meta-analytic studies included fewer participants than ours (e.g. the previous meta-analysis included around 2,350 children with ADHD for analysis of the 148-bp allele, and less than 1,000 patients for analysis of the 136-bp allele (Wu et al., 2012)), which could explain the discrepancy between findings. Only three earlier studies investigating the association between the *DRD5* VNTR and persistent ADHD have been published (Carpentier et al., 2013; Johansson et al., 2008; Squassina et al., 2008), and one additional study used a childhood cohort that was followed-up for five years (Langley et al., 2009). The results were inconsistent across studies, with only two studies reporting some evidence of positive association (Johansson et al., 2008; Langley et al., 2009); one of those two was a subsample of the Norwegian sample included in the current study, and the originally reported association was no longer present in this larger sample. In conclusion, based on the results of the current meta-analysis, it seems unlikely that the *DRD5* VNTR contributes substantially, if at all, to ADHD persistence into adulthood.

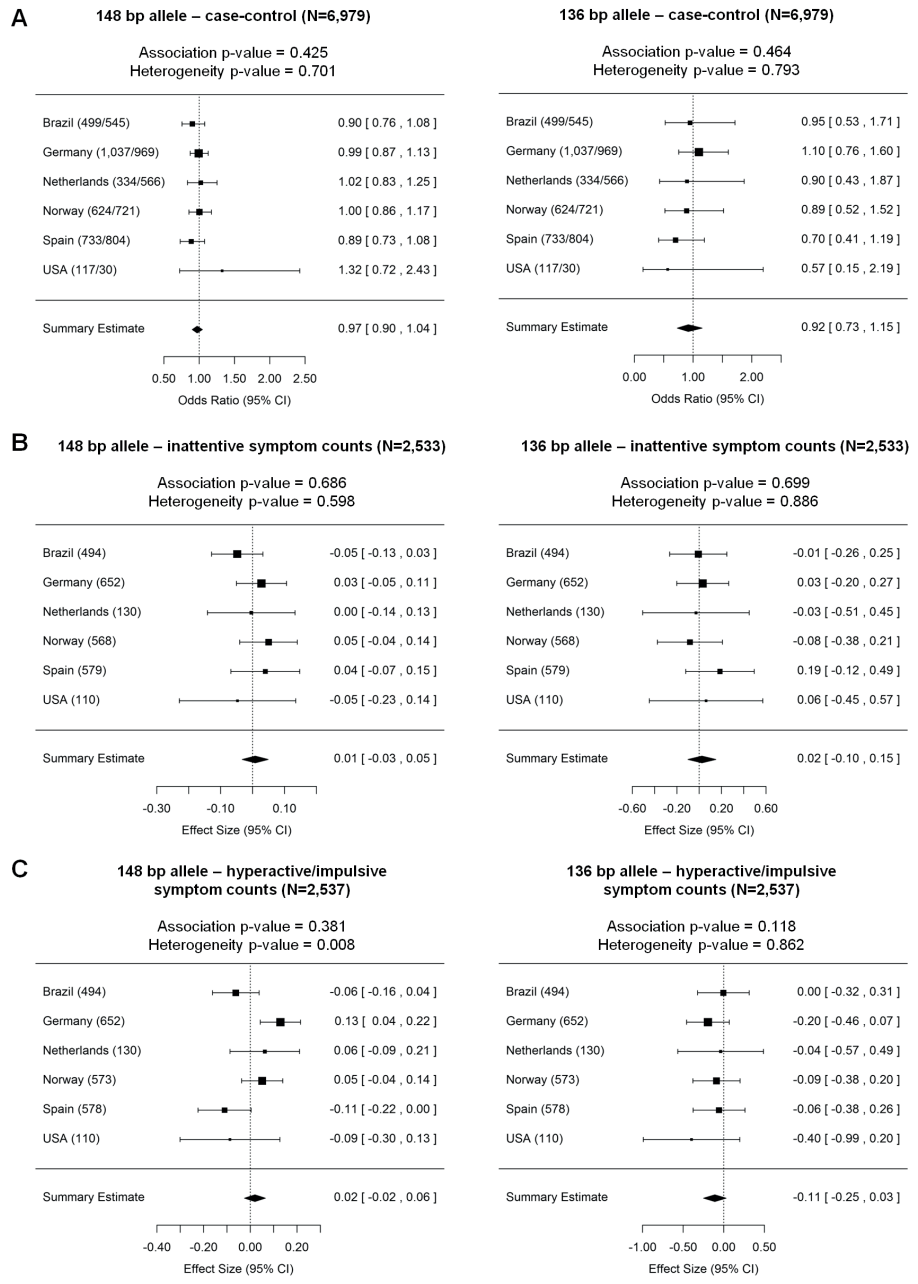

Figure 2: (**A**) Forest plots showing the association analysis of the *DRD5* 148-bp and 136-bp alleles with ADHD risk. (**B**) Forest plots showing the association analysis of the *DRD5* 148-bp and 136-bp alleles with ADHD inattentive symptom counts in patients with adult ADHD. (**C**) Forest plots showing the association analysis of the *DRD5* 148-bp and 136-bp alleles with ADHD hyperactive/impulsive symptom counts in patients with adult ADHD. For each, cohort the number of cases and controls is presented in brackets.

Acknowledgements

The authors wish to thank all participants who took part in the study. This study is part of the International Multicentre persistent ADHD Collaboration (IMpACT; www.impactadhdgenomics.com). IMpACT unites major research centers working on the genetics of ADHD persistence across the lifespan and has participants in the Netherlands, Germany, Spain, Norway, the United Kingdom, the United States, Brazil, and Sweden. Principal investigators of IMpACT are: Barbara Franke (chair), Andreas Reif (co-chair), Stephen V. Faraone, Jan Haavik, Bru Cormand, Antoni Ramos-Quiroga, Philip Asherson, Klaus-Peter Lesch, Jonna Kuntsi, Claiton H. D. Bau, Jan Buitelaar, Stefan Johansson, Henrik Larsson, Alysa Doyle, and Eugenio H. Grevet. Part of the Dutch controls was derived from the Nijmegen Biomedical Study. Principal investigators of the Nijmegen Biomedical Study are LALM Kiemeney, ALM Verbeek, DW Swinkels, and B Franke.

Conflict of interest

Dr. Ramos-Quiroga has served on the speakers' bureau and acted as consultant for Eli Lilly and Co., Janssen-Cilag, Novartis, Lundbeck, Shire, Ferrer, and Laboratorios Rubió. He has received travel awards from Eli Lilly and Co., Janssen-Cilag, and Shire for participating in psychiatric meetings. The ADHD Program chaired by Dr. Ramos-Quiroga has received unrestricted educational and research support from Eli Lilly and Co., Janssen-Cilag, Shire, Rovi, and Laboratorios Rubió in the past two years.

Dr. Grevet was on the speaker's bureau for Novartis and Shire for the last 3 years. He also received travel awards (air tickets and hotel accommodations) for participating in two psychiatric meetings from Shire and Novartis. Dr. SV Faraone has received research support from, served as consultant or adviser to or participated in CME programmes sponsored by Alcobra, Janssen, Eli Lilly, NIH, Novartis, McNeil, Pfizer and Shire; he receives royalties from Guilford Press and Oxford University Press. In the

past year, Dr. SV Faraone received income, potential income, travel expenses and/or research support from Pfizer, Ironshore, Shire, Akili Interactive Labs, CogCubed, Alcobra, VAYA Pharma, Neurovance, Impax and NeuroLifeSciences. With his institution, he has US patent US20130217707 A1 for the use of sodium-hydrogen exchange inhibitors in the treatment of ADHD. All other authors declare that they have no conflicts of interest.

Contributors

MK, AAV, and BF conceived and designed the study. MH, JD, RM, AJGAMH, TEG, HW, SKS, MR, TZ, TAH, KKJ, NRM, EHG, and AD contributed data. MK and SB conducted statistical analyses and wrote the first draft of the manuscript. BF, LALMK, KPL, AR, JAR, BC, SJ, JH, CHDB, and SVF provided funding for the project. All co-authors provided critical feedback on the manuscript, suggested additional analyses and critical revisions, and edited the manuscript for clarity and precision. All authors contributed to and have approved the final manuscript.

Role of Funding Source

IMpACT-NL: The Dutch IMpACT study is supported by grants from the Netherlands Organization for Scientific Research (NWO), i.e. the NWO Brain & Cognition Excellence Program (grant 433-09-229) and a Vici grant to BF (grant 016-130-669), and by grants from the Netherlands Brain Foundation (grant 15F07[16]27) and BBMRI-NL (grant CP2010-33). [2]27) and BBMRI-NL (grant CP2010-33). The German IMpACT study is supported b grants from the DFG (KFO 125 to AR and KPL; SFB TRR 58 to AR and KPL; RTG 1252, to AR and KPL), BMBF 01GV0605, to KPL).

IMpACT-SPAIN: The Spanish IMpACT study is supported by Instituto de Salud Carlos III (PI11/00571, PI11/01629, PI12/01139, PI14/01700), and cofinanced by the European Regional Development Fund (ERDF), Agència de Gestió d'Ajuts Universitaris i de Recerca-AGAUR, Generalitat de Catalunya (2014SGR1357, 2014SGR0932), Ministerio de Economía y Competitividad, Spain (SAF2012-33484) and a NARSAD Young Investigator Grant from the Brain & Behavior Research Foundation. M Ribasés is a recipient of a Miguel de Servet contract from the Instituto de Salud Carlos III, Spain (CP09/00119).

IMpACT-BRA: Claiton H. D. Bau received the following grants from the Brazilian National Council for Scientific and Technological Development (CNPq): 476529/2012-3; 306678/2012-8; 466722/2014-1. The research leading to these results also received funding from the European Community's Seventh Framework Programme (FP7/2007 – 2013) under grant agreement n° 602805 (Aggressotype) and from the European Community's Horizon 2020 Programme (H2020/2014 – 2020) under grant agreement n° 643051 (MiND).

None of the funding sources had further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

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CHAPTER 3

STXBP5 Antisense RNA 1 gene and adult ADHD symptoms

In revision as:

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Abstract

Attention-deficit/hyperactivity disorder (ADHD) is characterized by age-inappropriate levels of inattention and/or hyperactivity-impulsivity and persists into adulthood in a substantial proportion of cases. ADHD is heritable, and is thought to represent the clinical extreme of a continuous distribution of ADHD symptoms in the general population. We aimed to detect ADHD risk conferring genes leveraging the power of population studies of ADHD symptoms in adults. Within the SAGA (Study of ADHD trait Genetics in Adults) consortium, we estimated the SNP-based heritability of self-reported ADHD symptoms and carried out a genome-wide association meta-analysis in nine adult population-based and case-only cohorts of unrelated adults. A total of n=14,689 individuals were included. We found a significant SNP-based heritability for self-rated ADHD symptom scores of respectively 15% (n=3,656) and 30% (n=1,841) in the two cohorts. The top-hit of the genome-wide meta-analysis (SNP rs12661753) was present in the hitherto uncharacterized long non-coding RNA *STXBP5-AS1* gene. This association was also observed in a meta-analysis of childhood ADHD symptom scores in eight population-based pediatric cohorts from the EAGLE ADHD consortium (n=14,776). Genome-wide meta-analysis of the SAGA and EAGLE data (n=29,465) increased the strength of the association on the *STXBP5-AS1* gene. In human HEK293 cells, expression of *STXBP5-AS1* enhanced the expression of a reporter-construct of *STXBP5*, a gene known to be involved in SNARE complex formation. In mouse strains featuring different levels of impulsivity, *Stxbp5-AS1* transcript levels in the prefrontal cortex strongly correlated with motor impulsivity as measured in the 5-choice serial reaction time task (r^2=0.55). Our results implicate the *STXBP5-AS1* gene in ADHD symptom scores and point to vesicle transport as a biological mechanism involved in ADHD-related impulsivity levels.

Key words: ADHD behavior, Study of ADHD trait Genetics in Adults, *STXBP5-AS1* gene, vesicle transport in ADHD

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a common neurodevelopmental disorder affecting 2–5% of children (Polanczyk and Rohde, 2007)[-](Association, 1994) and adults (Franke et al., 2012)[,](Faraone et al., 2015). ADHD is characterized by age-inappropriate, sustained symptoms of inattention and/or hyperactivity-impulsivity. In children (Faraone et al., 2005) and adults (Saviouk et al., 2011), ADHD shows substantial heritability. Heritability estimates are largely independent of the phenotypic measurement scale (i.e., categorical or continuous) in children; in adults, estimates are lower when using self-report rating scales (Franke et al., 2012). Twin studies (Faraone et al., 2005) suggest that etiological influences on ADHD symptoms are distributed throughout the population, consistent with a liability model (Caspi et al., 2008). Inattention and hyperactivity-impulsivity symptoms can be reliably assessed in population-based cohorts based on rating scales (Larsson et al., 2013), creating the possibility to collect large samples for gene-finding studies. The genetic contributions to ADHD in children and adults are complex, with multiple different genetic variants contributing to the disorder (Faraone et al., 2015), both common and rare (Franke et al., 2012). Recently, 16 genome-wide associations have been established in an ADHD Genome-Wide Association Studies meta-analysis (GWASMA) of childhood case-control studies from the Psychiatric Genomics Consortium (PGC) and The Lundbeck Foundation Initiative for Integrative Psychiatric Research (iPSYCH)[9] and population-based samples from the EArly Genetics and Lifecourse Epidemiology (EAGLE) consortium (Demontis et al., 2017; Middeldorp et al., 2016).

Here, we sought to leverage the power of population studies of ADHD symptoms in adults to detect disease-relevant genes. Within the SAGA (Study of ADHD trait Genetics in Adults) consortium, we estimated the SNP-based heritability of self-reported adult ADHD symptoms and subsequently carried out a GWASMA in nine cohorts of European Caucasian origin (n=14,689 individuals, age 18 years or older), in whom adult self-reported ADHD symptom scores were available. These samples included six population-based cohorts, two clinical ADHD samples and one clinical cohort ascertained for depressive and anxiety disorders (to enrich the clinical extreme of the ADHD symptom continuum). The locus with the strongest statistical association was followed-up in a replication analysis of quantitative childhood ADHD symptom scores (n=14,776) from the EAGLE consortium (Middeldorp et al., 2016). Genetic correlations were obtained between the PGC and the iPSYCH sample of children (Demontis et al., 2017) and the SAGA sample of adults. Finally, we conducted gene-based tests for genes with SNPs showing a P value<$1x10^{-6}$ in the meta-analysis, making use of the common SNPs from SAGA and rare variant data from the Erasmus Rucphen Family (ERF) study (see **Table 1**), one of the adult cohorts.

Functional follow-up studies downstream of gene-finding in ADHD, e.g. in model systems, to determine the biological relevance of a genetic finding, are scarce (Klein et al., 2017). Core features of ADHD, inattention, hyperactivity, and impulsivity are well defined e.g. in mouse models (Loos et al., 2014). Here we carried out functional follow-up studies for the hitherto uncharacterized top-gene of the GWASMA in three mouse inbred strains with large differences in motor impulsivity derived from reaction time tasks, and in a human cell assay.

Methods

ADHD symptom scores and study populations

ADHD symptom scores were assessed by three instruments (see **Table 1**) in nine cohorts (for a complete description of each sample please follow the references in **Table 1**): the ADHD-index of the Conners Adult ADHD Rating Scale (Conners, 1999) (CAARS ADHD-index; 12 items), the total scores of the DSM-IV ADHD Rating Scale (ADHD-RS) (Sandra Kooij et al., 2008), and the Attentional Deficit/Hyperactivity Problems subscale from the Adult Self Report (ASR-ADHD; 13 items) (Kessler et al., 2007). The CAARS (used in NESDA, NTR, ERF) is an extensively tested psychometric instrument with high internal consistency and reliability. Five cohorts (NeuroIMAGE, BIG, IMpACT-NL, VHIR, NBS) collected information using the ADHD-RS (Sandra Kooij et al., 2008), which has high validity in population-based and case samples. For IMpACT-NL and VHIR, only affected individuals were included. One cohort (TRAILS) assessed ADHD problems through the ASR-ADHD (Kessler et al., 2005; Kessler et al., 2007)·(Achenbach, 2003) (http://www.aseba.org/).

Genetic Variant Calling and Quality Control

An overview of genome-wide single nucleotide polymorphism (SNP; for common variants) genotyping, quality control, and imputation is given in **Supplementary Table 1**. Exomes of 1,336 individuals from the ERF population, which is a genetically isolated population in the Netherlands (Aulchenko et al., 2004), were sequenced (see **Supplementary Methods**), and ADHD index data were available for 587 of these individuals. Detection of rare variants in the ERF study was done for those genes with SNPs with $P<1x10^{-5}$ in the GWASMA, and variants identified in these exomes were used to estimate the contribution of rare variants in the genes of interest to ADHD behavior (see **Supplementary Methods**).

GCTA

Genome-wide Complex Trait Analysis (GCTA) (Yang et al., 2011) was used to compute the variance in the ADHD symptom score explained by common SNPs in the two largest cohorts included in the meta-analysis, the NTR and NESDA (n>1,500 unrelated subjects). A genetic relationship matrix (GRM) for all individuals in the dataset was estimated based on SNPs

with high imputation quality (see **Supplementary Methods**). Bivariate GCTA (Yang et al., 2011) was additionally run on the ADHD-index of the CAARS and ASR-ADHD data also available in the NTR cohort, to assess the genetic correlation (r_g) between the two diagnostic instruments.

Genome-wide association and Meta-analysis

GWAS was conducted in each cohort by linear regression under an additive model. Age was included as a covariate, but not gender, which was not significantly associated with the ADHD scores in any study. Four principal components were added to account for possible population stratification effects. Information on software packages is provided in **Supplementary Table 1**. In all analyses, the uncertainty of the imputed genotypes was taken into account. Location of SNPs reported is from the build 37 (hg19) 1000G data. Meta-analysis was conducted in METAL (www.sph.umich.edu/csg/abecasis/metal/index.html) by the *P*-value-based method, given the intrinsic variability of the quantitative traits used (see **Supplementary Methods**). The meta-analysis was performed in the full sample (nine cohorts) and restricted to the population-based samples (seven cohorts; "restricted sample").

Replication in the EAGLE consortium

Within EAGLE, association of ADHD-related measures was assessed in nine population-based childhood cohorts with genotype data imputed against the 1000 Genomes reference panel (Middeldorp et al., 2016). Linear regression of the phenotype on sex, age, genotype dose, and principal components was performed in all cohorts, followed by meta-analysis based on *P*-values in METAL. The TRAILS cohort is part of both consortia, and was excluded from the EAGLE consortium for replication analysis, leaving a total of 14,776 children from eight cohorts.

Look-up of significant GWAS loci

Evidence for an effect of the 12 independent ADHD-associated SNPs from the PGC+iPSYCH GWASMA on adult ADHD symptoms was studied through a look-up of results. LD-independent loci with corresponding index-SNPs were obtained from Table 1 of Demontis et al (Demontis et al., 2017). If the index variant was not present in the SAGA data set, a proxy variant was selected using LDlink (https://analysistools.nci.nih.gov/LDlink/). The Bonferroni-corrected significance level was set at $P=0.05/12=0.00417$.

Linkage disequilibrium score regression (LDSR) analysis

LDSR was used to estimate the genetic correlation between the PGC+iPSYCH sample of children (Demontis et al., 2017) and the SAGA sample of adults. Each dataset underwent additional filtering for markers overlapping with HapMap Project Phase 3 SNPs, INFO

score≥0.9 (where available), and MAF≥1%. Indels and strand-ambiguous SNPs were removed. LDSR analysis was performed using the LDSR package (https://github.com/bulik/ldsc (Bulik-Sullivan et al., 2015), see **Supplementary Methods**).

Gene-wide analysis of common and rare variants

Genes containing SNPs with *P*-values<1x10^{-6} in the meta-analysis of the nine cohorts were selected for gene-wide tests using common and rare variants. The common variant analysis was performed in MAGMA (de Leeuw et al., 2015). Flanking regions of 25kb for each gene were included in the analyses. The rare variant analysis was performed with the Sequence Kernel Association Test (SKAT; only in the ERF study) library of the R-software (Wu et al., 2011).

Functional analyses

Follow-up functional analyses were performed on the locus containing the best association *P*-value. This locus contains *STXBP5-AS1*, representing a putative long noncoding RNA, predicted to be expressed in several species (**Supplementary Methods; Supplementary Table 5 & Supplementary Fig. 2**). Human *STXBP5-AS1* encodes multiple splice variants, many of which lack a region that overlaps the *STXBP5* gene. To test for regulatory effects of *STXBP5-AS1* on the expression of *STXBP5*, a fluorescent reporter construct was designed to contain the region of antisense overlap (see **Supplementary Methods**).

Mouse models

RNA was derived from prefrontal cortex of adult male mice from the inbred strain C57/Bl6J (n=7) and recombinant inbred strains BXD29 (n=8) and BXD68 (n=7), and gene expression was quantitated (see **Supplementary Methods**). Strains were bred in the facility of the Neuro-Bsik consortium of the VU University (Amsterdam, The Netherlands) and used for behavioral analysis (Loos et al., 2014)(Spijker et al., 2004).

Results

Quantitative assessment instruments are listed in **Table 1**. The quantitative phenotypes showed a weak, negative correlation with age and no association with gender in any cohort. Phenotypic and genetic correlations between symptom scores assessed with the different instruments were substantial: in a clinical sample of 120 adults with ADHD the phenotypic correlation between the CAARS (Conners, 1999) (ADHD-index) and the ADHD-RS (Sandra Kooij et al., 2008) (total score) was high (r=0.73; *P*<0.01) (Sandra Kooij et al., 2008). In 380 parents of children with ADHD, the correlation was of similar magnitude (r=0.69; *P*<0.001) (Thissen et al., 2012). We estimated the phenotypic correlation between the CAARS ADHD-index and the ASR-ADHD (Kessler et al., 2005; Kessler et al., 2007) in the NTR (n=15,226;

Table 1. Descriptive information for all cohorts and for phenotype assessment in the SAGA consortium.

Cohort Name	N (% F)	Age (SD)	Symptom list (N items)	Score range*	Mean Score (SD)*	Ref
NTR	5935 (63%)	43.7 (15.2)	CAARS ADHD-index (12)	0–30	7.9 (3.7)	(Willemsen et al., 2010)
NESDA	1977 (66%)	46.5 (13.0)	CAARS ADHD-index (12)	0–32	8.7 (5.4)	(Boomsma et al., 2008)
ERF	1043 (53%)	45.6 (13.3)	CAARS ADHD-index (12)	0–25	7.8 (4.4)	(Aulchenko et al., 2004)
NeuroIMAGE	470 (51%)	42.3 (5.3)	ADHD-RS (23)	0–43	14.1 (8.9)	(von Rhein et al., 2014)
BIG	448 (63%)	22.3 (3.2)	ADHD-RS (23)	0–40	14.0 (6.4)	(Hoogman et al., 2012)
NBS	2925 (53%)	57.4 (16.3)	ADHD-RS (23)	0–15	1.4 (2.2)	(Galesloot et al., 2017)
IMpACT-NL±	113 (62.8%)	37.7 (11.5)	ADHD-RS (23)	1–18	12.04 (3.3)	(Franke and Reif, 2013)
VHIR±	559 (32%)	33.3 (10.6)	ADHD-RS (18)	4–54	31.0 (9.7)	(Bosch R, 2009)
TRAILS	1215 (48%)	19.0 (0.6)	ASR ADHD (13)	0–22	5.9 (4.4)	(Ormel et al., 2014)

Conners' Adult ADHD Rating Scale (CAARS ADHD-index), DSM-IV ADHD Rating Scale (ADHD-RS), and Attentional Deficit/Hyperactivity Problems subscale from the ASR (ASR ADHD); *Untransformed values observed per cohort; ± only affected individuals included.

average age 40 years, SD=16.1) to be 0.67 (*P*<0.0001). In younger participants in the age range of the TRAILS cohort (18–22 years, n=2,687), the correlation was similar (0.68, *P*<0.0001).

A significant SNP-based heritability was estimated for the CAARS ADHD-index in a subsample of each of the two largest cohorts: 30% (SE=16.7%, *P*=0.035) in NESDA (n=1,841 unrelated subjects) and 15% (SE=7.8%, *P*=0.020) in NTR (n=3,881 unrelated participants). We also estimated the genetic correlation for the CAARS ADHD-index and the ASR-ADHD using bivariate GCTA. In all individuals from the NTR with genotype and phenotype data (n=6,036 related and unrelated subjects), the genetic correlation was 0.818 (SE=0.256). When analyzing the bivariate data in 2,921 unrelated subjects, the point estimate of the genetic correlation was 0.813 (SE=0.364). The significant SNP-based heritability and the considerable phenotypic and genetic correlations between assessment instruments support the validity of our meta-analysis approach of GWA results obtained across contributing data sets.

For the nine separate GWAS, the genomic control inflation factors (lambda) ranged between 0.996 and 1.026 (mean lambda 1.009, **Supplementary Table 2**). Meta-analysis (**Supplementary Figure 1A**) of the full sample revealed the lowest *P*-value ($3.03x10^{-7}$) for the intronic SNP rs12661753 in *STXBP5-AS1* (**Supplementary Figure 3**); for the meta-analysis of the restricted sample, *P*-value for this SNP was $1.48x10^{-6}$ (**Supplementary Figure 1B**). Replication was observed for rs12661753 (*P*=$3.07x10^{-2}$) for childhood ADHD symptoms in the EAGLE-ADHD consortium (Middeldorp et al., 2016). The subsequent GWASMA between SAGA and EAGLE revealed the best association *P*-value=$2.05x10^{-7}$ for SNP rs12664716 (n=29,465; **Supplementary Figure 3F**) located in the *STXBP5-AS1* gene, and in high LD (D'=1.0, r^2=0.98) with rs12661753 ($P_{SAGA-EAGLE}$= $3.55x10^{-7}$; **Supplementary Figure 1C**).

The index variant rs12661753 was not associated with ADHD risk in the recent case-control PGC+iPSYCH GWASMA of ADHD in a sample mainly consisting of children (*P*=0.6316, n=55,374). A look-up of genome-wide significant ADHD index SNPs from this PGC+iPSYCH GWASMA for association in the SAGA consortium also revealed no significant associations with adult ADHD symptoms (**Supplementary Table 6**).

We estimated the genetic correlation between PGC+iPSYCH and the complete SAGA sample to be 0.541 (SE=0.447, *P*=$2.26x10^{-1}$; the VHIR cohort present in both studies). We tested if the two r_g values differed significantly from each other, which was not the case (X^2-based test *P*>0.05) (**Supplementary Methods**).

In NTR and NESDA, a subset of participants (n=6,678) had additional phenotype data on hyperactivity/impulsivity and inattention symptom subscales of the CAARS available. These scales of each 9 items are non-overlapping with the 12 ADHD-index items. For hyperactivity/impulsivity symptoms, the *P*-value for association with rs12661753 was $1.51x10^{-5}$, whereas for inattention it was $3.53x10^{-2}$, suggesting a stronger effect of the variant on hyperactivity/impulsivity.

As shown in **Table 2** and **Supplementary Table 3**, 50 common variants from 8 independent (clumped) loci showed *P*-values<$1x10^{-6}$. Of these, four were also amongst the top-associated loci from the restricted SAGA GWASMA (no patients; **Supplementary Table 4**). The genes closest to these SNPs were selected for gene-wide analysis (**Table 2**). Analysis of common variants in seven genes (plus 25kb flanking regions) in the SAGA GWASMA showed significant association with ADHD symptoms. Two significant findings (P<0.003) were for long intergenic non-protein coding RNA genes (*LINC01247*, *LINC00534*), and nominal significant associations (*P*<0.05 gene-wide) were seen for *STXBP5-AS1*, *CALB1*, *GNG12-AS1*, *STXBP5* (**Supplementary Table 5**). It is important to note that *STXBP5* and *STXBP5-AS1* have no physical separation, thus their 25kb flanking regions overlap. The rare variant analysis also showed nominal association for *STXBP5*. For four genes (*GNG12-AS1, LINC01247, STXBP5-AS1, LINC00534)*, rare variants were not observed/detected ((**Supplementary Table 5**).

Given that the *STXBP5-AS1* gene, which contains the top-hits, is hitherto uncharacterized, we investigated its function. *STXBP5-AS1* encodes a long noncoding RNA (lncRNA). Although human *STXBP5-AS1* does not have any orthologues listed in the UniGene database, it is conserved in primates and shows a modest conservation in rodents (**Supplementary Table 7** and alignment in **Supplemental Figure 2**). In the hg19 genome release annotation *STXBP5-AS1* is located next to *STXBP5* in the opposite orientation, with antisense sequence overlap in exon 1 of *STXBP5* (**Figure 1A**). It may be hypothesized that *STXBP5-AS1* affects *STXBP5* expression. For such natural antisense RNAs, both repression and positive effects on the expression of cognate genes have been described (Kimura et al., 2013; Matsui et al., 2008). We tested this hypothesis by designing a reporter gene fusing exon 1 of *STXBP5* to *EGFP*, and quantifying its expression in human HEK293 cells. Expression of the antisense lncRNA variant *STXBP5-AS1-003* (containing the overlap with *Stxbp5*) caused an increase in the fluorescence ratio between STXBP5-EGFP and the control (**Figure 1B-E**).

Table 2. Most strongly associated (clumped) SNPs (*P*-value<1x10^{-6}) coming from the meta-analysis of nine cohorts from the SAGA consortium in physical position order (hg19).

SNP name	Chr	Locus	Pos*	p-value	Tested/Non-Tested Allele	Frequency Tested Allele	Gene(s) in locus
rs11209188	1	1p31.3	68455306	7.88x10^{-6}	A/G	0.534	*GNG12-AS1*
rs1930272	1	1p31.1	83491910	4.75 x10^{-6}	T/C	0.544	*LOC107985037*
rs1564034	2	2p25.2	6510305	2.15 x10^{-6}	T/G	0.670	*LINC01247*
rs28734069	4	4q26	120042409	5.77 x10^{-6}	T/C	0.016	*LOC102723967; LOC105377395*
rs12661753	6	6q24.3	147409235	3.02 x10^{-7}	A/G	0.962	*STXBP5-AS1*
rs13274695	8	8p23.2	3723378	6.00 x10^{-6}	A/G	0.077	*CSMD1; LOC105377790*
rs2189255	8	8q21.3	91190297	9.61 x10^{-6}	T/C	0.703	*CALB1; LINC00534*
rs73204517	13	13q21.33	69920315	7.19 x10^{-6}	C/G	0.126	Downstream *LINC00383*

*bp position based on the GRCh37.p13 build; • Allele frequency of tested allele based on N=14,689

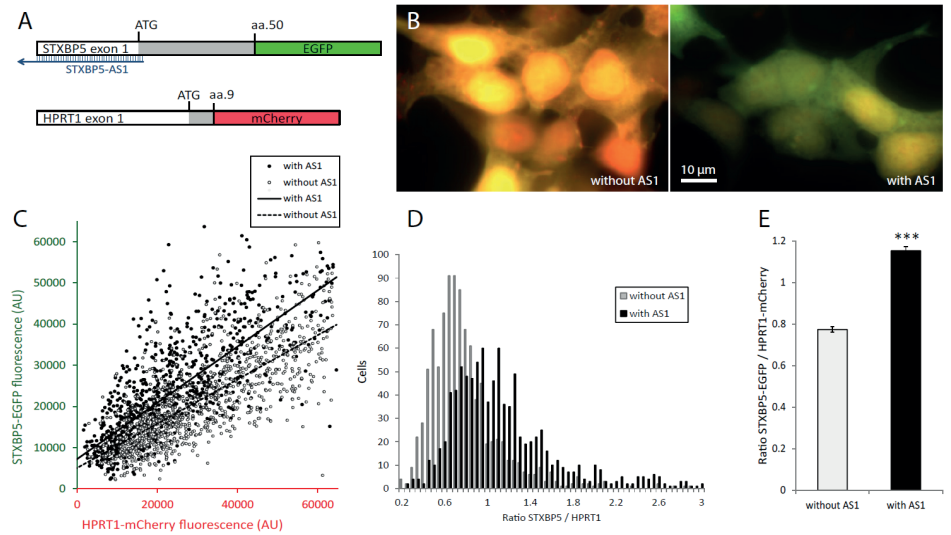

Figure 1. STXBP5-AS1 positively regulates the expression of its cognate mRNA. (**A**) Design of two reporter constructs. Top: Exon 1 of human *STXBP5*, containing the natural 5'UTR and encoding the first 50 amino acids, was fused in-frame to EGFP. The *Stxbp5-AS1* transcript including the region showing perfect (100%) sequence overlap with the encoded *Stxbp5* transcript is depicted schematically in blue. Bottom: To control for transfection efficiency and differences in cellular metabolic rates, we co-expressed a non-target mRNA comprised of human *HPRT1* exon 1 fused to mCherry. (**B**) Typical examples of HEK293 cells expressing both constructs with or without STXBP5-AS1. (**C**) Quantitation of EGFP and mCherry fluorescence in presence or absence of AS1 (947 and 974 cells respectively). (**D-E**) The ratio of STXBP5-EGFP and HPRT1-mCherry was calculated for each cell. Data are presented as a histogram (D) or as mean±SEM (E). ***, P=6x10^{-51}; t_{946}=4.4412, Student's t-test.

Given the *in vitro* effects on STXBP5-EGFP protein expression, we tested the relationship between gene expression of mouse *Stxbp5* and/or *Stxbp5-AS1* and measures of behavioral impulsivity. We analyzed gene expression in medial prefrontal cortex of three mouse inbred strains previously described to have large differences in motor impulsivity(Loos et al., 2014). Here, we confirmed the strain difference in motor impulsivity between the BXD68, BXD29, and C57BL/6J strains ($F_{2,20}$=6.91, P=0.005), measured as premature responses in the 5-choice serial reaction time task. In addition, these strains showed differences in errors of omission ($F_{2,20}$=5.18, P=0.015), but not attention ($F_{2,20}$=0.35, P=0.771) (**Figure 2A**). In these mice, we detected expression of a mouse *Stxbp5-AS1* transcript in the prefrontal cortex by real-time quantitative PCR, which differed across strains ($F_{2,19}$=11.73; P<0.001). This transcript showed lowest expression in the most highly impulsive strain, BXD68 (BXD68: 4.58±0.11, C57BL/6J: 5.25±0.14, BXD29: 5.19±0.07, $P_{BXD68\ vs\ C57BL/6J}$=0.003, t_{13}=3.73; $P_{BXD68\ vs\ BXD29}$<0.001, t_{14}=4.63) (**Figure 2B**). Expression of *Stxbp5* mRNA was not different between the three strains (BXD68: 9.89±0.24; C57BL/6J: 9.83±0.10; BXD29: 9.99±0.23). These results suggest that the role of *STXBP5-AS1* plays in impulsivity is not due to influencing the level of the *STXBP5* transcript. Examining correlations between *Stxbp5-AS1* transcript level and impulsivity/inattention measures, we found a significant correlation with motor impulsivity (r^2=0.55; P=8.26x10^{-5},

Bonferroni-corrected *P*<0.0083) and a nominally significant association with attention, when measured as errors of omission (Guillem et al., 2011) (r^2=0.1765; *P*=5.16x10^{-2}), but not when measured as percentage correct responses (r^2=0.0862; *P*=1.85x10^{-1}). Expression of *Stxbp5* did not correlate with these parameters (**Figure 2C**).

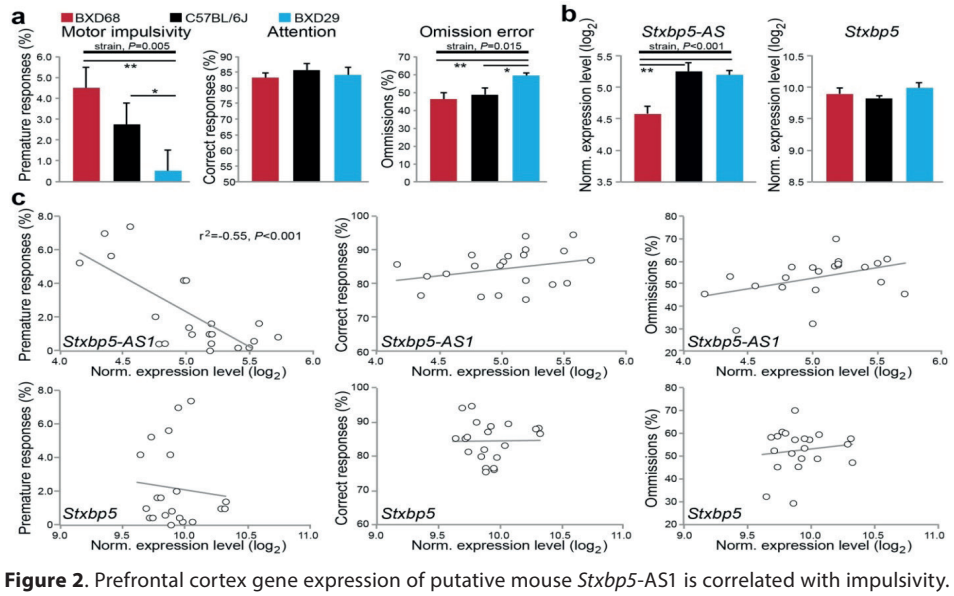

Figure 2. Prefrontal cortex gene expression of putative mouse *Stxbp5*-AS1 is correlated with impulsivity. (**A**) Mouse strains BXD68 (red, n=7), C57BL/6J (black, n=8) and BXD29 (blue, n=8) were selected based on a difference in premature responses (motor impulsivity; BXD68 *vs.* BXD29, t_{14}=3.71; C57BL/6J *vs.* BXD29, t_{15}=2.78) and error of omissions (BXD68 *vs.* BXD29, t_{14}=3.54; C57BL/6J *vs.* BXD29, t_{15}=2.52), without being different on accuracy (Loos et al., 2014). Shown are data (mean±SEM) of the animals used for gene expression analysis (see b). (**B**) Strain mean±SEM of prefrontal cortex gene expression in BXD68 (red, n=7), C57 (black, n=7), and BXD29 (blue, n=8) for *Stxbp5-AS1* (left) and *Stxbp5* (right). *Stxbp5-AS1* is differentially expressed between strains, with lower expression in BXD68. Yet, *Stxbp5* shows no differential expression. No difference in variation was observed. (**C**) Gene expression of *Stxbp5-AS1* (upper panels) in individual mice for which behavioral data was available (BXD68, n=6; C57BL/6J, n=7; BXD29, n=8) correlated well with premature responses (motor impulsivity; left), not with accuracy (attention; middle), and showed a trend towards correlation with errors of omissions (right; *P*=0.0516). *Stxbp5* expression (lower panels) did not correlate with any of these parameters. Trend lines are given in gray. * *P*<0.05; ** *P*<0.01; ***P<0.001.

Discussion

We report a genetic variant associated with three different but correlated adult ADHD symptom lists in a meta-analysis of nine European adult population-based and case-only cohorts (n=14,689 individuals). The *STXBP5-AS1* gene (best SNP *P*=3.02x10^{-7}) was the most strongly associated locus in a meta-analysis. This association was confirmed in the EAGLE meta-analysis (P_{EAGLE}=2.89x10^{-2}), and the top-hit from the full SAGA-EAGLE GWASMA was

also located in the *STXBP5-AS1* gene and in almost perfect LD with the original finding (SNP rs12664716, $P_{SAGA-EAGLE}$=2.05x10^{-7}; n=29,465).

For the adult ADHD-index, an earlier large twin family study estimated total heritability at 30%, and common SNPs thus contain substantial information concerning its genetic variance[10]. SNP-based heritability analyses, which were ran prior to GWASMA, provided estimates of 15-30% explained variance of adult ADHD symptom scores in the general population. Such estimates are comparable with the estimates obtained for ADHD and four additional categorically defined psychiatric disorders (Cross-Disorder Group of the Psychiatric Genomics et al., 2013), providing rationale for a gene-finding enterprise for adult ADHD symptoms in the general population.

The function of the *STXBP5-AS1*-encoded lncRNA is currently unknown. *STXBP5-AS1* has been proposed as a prognostic biomarker for survival of cancer patients (Guo et al., 2016), but no information is available for its role in ADHD, related traits, or other psychiatric diseases. It overlaps in anti-sense with *STXBP5* encoding a protein involved in synaptic function by regulating neurotransmitter release through stimulating SNARE complex formation (Sakisaka et al., 2008)·(Yizhar et al., 2004). This complex plays a major role in intracellular vesicular trafficking in eukaryotic cells and is involved in the exocytotic release of neurotransmitters during synaptic transmission (Antonucci et al., 2016). Genes related to the SNARE complex and its regulators have been investigated in ADHD (Bonvicini et al., 2016), and current results suggest that this complex may exert distinct roles throughout development, with age-specific effects of its genetic variants on ADHD behavior (Cupertino et al., 2016). Specifically, deletions and mutations of *STXBP5* occur in autism (Davis et al., 2009) and epilepsy (Dhillon et al., 2011). *STXBP5* has a presynaptic role that negatively regulates neurotransmitter release by forming syntaxin-SNAP25-tomosyn complex (Sakisaka et al., 2004). However, the postsynaptic role of STXBP5 has not been well elucidated.

Post-hoc analysis suggested that *STXBP5-AS1* affects hyperactivity-impulsivity more strongly than inattention. The stronger link with impulsivity was corroborated in behavioral studies in mice. Our experiments in HEK293 cells showed that the lncRNA does not cause antisense inhibition of *Stxbp5*. The increased fluorescence of a reporter protein containing mouse *Stxbp5* exon 1, together with unchanged *Stxbp5* mRNA levels in mouse strains expressing different *Stxbp5-AS1* levels, suggest that the lncRNA might enhance Stxbp5 protein translation or stability. Alternatively, *Stxbp5-AS1* might contribute to impulsivity by a Stxbp5-unrelated mechanism. In line with this idea, *Stxbp5-AS1*, expression (but not that of *Stxbp5*) correlated negatively with motor impulsivity in mice.

Our study should be viewed in the light of some strengths and limitations. A pro was the sample size that could be achieved for quantitative data available through a population-based approach. Moreover, the functional analyses provided a very strong candidate associated with adult and childhood ADHD symptoms. A limitation of our study was the combination of three different phenotyping instruments, but given the strong phenotypic

and genetic correlations between the instruments, this might not have reduced power substantially.

The genetic correlation of PCG+iPSYCH with SAGA should be interpreted carefully because the standard error is high. The fact that the PCG+iPSYCH/SAGA rg (0.54), did not differ from the published rg estimate between the PCG+iPSYCH GWASMA and a GWAS of the 23andMe sample (0.65, SE=0.114) (Demontis et al., 2017) is encouraging but not unexpected given the low power to detect a difference. The estimated genetic correlation between the 23andMe and PGC+iPSYCH analyses was significant but lower than the genetic correlation of the EAGLE and PCG+iPSYCH childhood cohorts (rg=0.943, SE=0.204, P=3.65x10^{-6}) (Demontis et al., 2017). The ADHD diagnosis (yes/no) in 23andMe is based on the self-reported answer to a single question about presence of a lifetime diagnosis of ADHD (Demontis et al., 2017) and we do not know if the 23andMe participants were diagnosed in childhood or as adults. With a further increase in GWAS sample size update r_g results could suggest that there are different genetic correlation patterns between the association results estimated from the GWAS of adult (population-based) ADHD behavior and the GWAS from children, at this point the lack of power makes these analyses inconclusive.

Our study shows that self-reported adult ADHD symptoms measured in the general population have a genetic component and that performing population-based GWASMA of adult ADHD symptoms provides novel insights into the genetic underpinnings of hyperactivity/impulsivity symptoms that are a hallmark of ADHD. Our findings implicate synaptic function regulation through *STXBP5-AS1* and potentially *STXBP5* in ADHD symptom etiology.

Acknowledgments

1. This work was sponsored by the **Stichting Nationale Computerfaciliteiten** (**National Computing Facilities Foundation, NCF**) for the use of supercomputer facilities, with financial support from the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (Netherlands Organization for Scientific Research, NWO)
2. This work made use of the **BIG** (**Brain Imaging Genetics**) database, first established in Nijmegen, The Netherlands, in 2007. This resource is now part of Cognomics (www.cognomics.nl), a joint initiative by researchers of the Donders Centre for Cognitive Neuroimaging, the Human Genetics and Cognitive Neuroscience departments of the Radboud university medical center and the Max Planck Institute for Psycholinguistics in Nijmegen. The Cognomics Initiative is supported by the participating departments and centres and by external grants, i.e. the Biobanking and Biomolecular Resources Research Infrastructure (Netherlands) (BBMRI-NL), the Hersenstichting Nederland, and the Netherlands Organisation for Scientific Research (NWO). We wish to thank all persons who kindly participated in the BIG research. The research leading to these results also receives funding from the European Community's Seventh Framework Programme (FP7/2007– 2013) under grant agreements #602450 (IMAGEMEND) and #602805 (Aggressotype), and from ERC-2010-AdG 268800-NEUROSCHEMA. In addition, we are funded by a grant from the National Institutes of Health (NIH) for the ENIGMA Consortium (Consortium grant U54 EB020403), supported by a cross-NIH alliance that funds Big Data to Knowledge Centers of Excellence.
3. The **Erasmus Rucphen Family study** (**ERF**) study as a part of EUROSPAN (European Special Populations Research Network) was supported by European Commission FP6 STRP grant number 018947 (LSHG-CT-2006-01947) and also received funding from the European Community's Seventh Framework Program (FP7/2007–2013)/grant agreement HEALTH-F4-2007-201413 by the European Commission under the program 'Quality of Life and Management of the Living Resources' of 5th Framework Program (no. QLG2-CT-2002-01254). High-throughput analysis of the ERF data was supported by joint grant from Netherlands Organization for Scientific Research and the Russian Foundation for Basic Research (NWO-RFBR 047.017.043). Exome sequencing analysis in ERF was supported by the ZonMw grant (project 91111025). Najaf Amin is supported by the Hersenstichting Nederland (project number F2013(1)-28). We are grateful to all study participants and their relatives, general practitioners and neurologists for their contributions and to P Veraart for her help in genealogy, J Vergeer for the supervision of the laboratory work, P Snijders for his help in data collection and E.M. van Leeuwen for genetic imputation.
4. The **International Multicentre persistent ADHD CollaboraTion** (**IMpACT**). IMpACT unites major research centres working on the genetics of ADHD persistence across the lifespan and has participants in the Netherlands, Germany, Spain, Norway, the United Kingdom, the United States, Brazil and Sweden. Principal investigators of IMpACT are: Barbara Franke (chair), Andreas Reif, Stephen V. Faraone, Jan Haavik, Bru Cormand, Antoni Ramos Quiroga, Philip Asherson, Klaus-Peter Lesch, Jonna Kuntsi, Claiton Bau, Jan Buitelaar, Stefan Johansson, Henrik Larsson, Alysa Doyle, and Eugenio Grevet. The Dutch IMpACT study is supported by grants from the Netherlands Organization for Scientific Research (NWO), i.e. the NWO Brain & Cognition Excellence Program (grant 433-09-229) and a Vici grant to BF (grant 016-130-669), and by grants from the Netherlands Brain Foundation (grant 15F07[2]27) and BBMRI-NL (grant CP2010-33). The research leading to these results also received funding from the European Community's Seventh Framework Programme (FP7/2007 – 2013) under grant agreements n° 602805 (Aggressotype), n° 278948 (TACTICS), and n° 602450 (IMAGEMEND), and from the European Community's Horizon 2020 Programme (H2020/2014 – 2020) under grant agreement n° 643051 (MiND) and n° 667302 (CoCA).

5. For the **Netherlands Study of Depression and Anxiety** (**NESDA**), funding was obtained from the Netherlands Organization for Scientific Research (Geestkracht program grant 10-000-1002); the Center for Medical Systems Biology (CSMB, NWO Genomics), Biobanking and Biomolecular Resources Research Infrastructure (BBMRI-NL), VU University's Institutes for Health and Care Research (EMGO+) and Neuroscience Campus Amsterdam, University Medical Center Groningen, Leiden University Medical Center, National Institutes of Health (NIH, R01D0042157-01A, MH081802, Grand Opportunity grants 1RC2 MH089951 and 1RC2 MH089995). Part of the genotyping and analyses were funded by the Genetic Association Information Network (GAIN) of the Foundation for the National Institutes of Health. Computing was supported by BiG Grid, the Dutch e-Science Grid, which is financially supported by NWO.
6. The **NeuroIMAGE** study was supported by grants from The Netherlands Organization for Health Research and Development (ZonMw 60-60600-97-193), the Netherlands Organization for Scientific Research (NWO, grants 1750102007010, 433-09-242 and 056-13-015), and by the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement number 278948 (TACTICS), 602450 (IMAGEMEND), 602805 (AGGRESSOTYPE), 603016 (MATRICS), and Horizon 2020 (grant agreements 643051 (MiND) and 642996 (BRAINVIEW)) research programs.
7. The **Nijmegen Biomedical Study** (**NBS**) is a population-based survey conducted at the Department for Health Evidence, and the Department of Laboratory Medicine of the Radboud university medical center. Principal investigators of the Nijmegen Biomedical Study are L.A.L.M. Kiemeney, A.L.M. Verbeek, D.W. Swinkels and B. Franke.
8. **NTR Research** was funded by the Netherlands Organization for Scientific Research (NWO: MagW/ZonMW grants 904-61-090, 985-10-002,904-61-193,480-04-004, 400-05-717, 91210020, Addiction-31160008, Middelgroot-911-09-032, Spinozapremie 56-464-14192 and NWO 480-15-001/674), Center for Medical Systems Biology (CSMB, NWO Genomics), NBIC/BioAssist/RK (2008.024), Biobanking and Biomolecular Resources Research Infrastructure (BBMRI-NL, 184.021.007), and the European Science Council (230374 and 284167). We thank the Avera Institute of Human Genetics for genotyping and technical support. DIB acknowledges the Royal Netherlands Academy of Science Professor Award (PAH/6635) We thank all participants in the Netherlands Twin Register.
9. The **TRacking Adolescents' Individual Lives Survey** (**TRAILS**) is a collaborative project involving various departments of the University Medical Center and University of Groningen, the Erasmus University Medical Center Rotterdam, the University of Utrecht, the Radboud Medical Center Nijmegen, and the Parnassia Bavo group, all in the Netherlands. TRAILS has been financially supported by grants from the Netherlands Organization for Scientific Research NWO (Medical Research Council program grant GB-MW 940-38-011; ZonMW Brainpower grant 100-001-004; ZonMw Risk Behavior and Dependence grant 60-60600-97-118; ZonMw Culture and Health grant 261-98-710; Social Sciences Council medium-sized investment grants GB-MaGW 480-01-006 and GB-MaGW 480-07-001; Social Sciences Council project grants GB-MaGW 452-04-314 and GB-MaGW 452-06-004; NWO large-sized investment grant 175.010.2003.005; NWO Longitudinal Survey and Panel Funding 481-08-013); the Dutch Ministry of Justice (WODC), the European Science Foundation (EuroSTRESS project FP-006), Biobanking and Biomolecular Resources Research Infrastructure BBMRI-NL (CP 32), the participating universities, and Accare Center for Child and Adolescent Psychiatry. We are grateful to all adolescents, their parents and teachers who participated in this research and to everyone who worked on this project and made it possible. Statistical analyses were carried out on the Genetic Cluster Computer (http://www.geneticcluster.org), which is financially supported by the Netherlands Scientific Organization (NWO 480-05-003) along with a supplement from the Dutch Brain Foundation.
10. The **Vall d'Hebron Research Institute** (**VHIR**) received financial support from "Fundació La Marató de TV3" (ref. 092330/31), "Instituto de Salud Carlos III-FIS" (PI11/00571, PI11/01629, PI12/01139, PI14/01700), "Agència de Gestió d'Ajuts Universitaris i de Recerca-AGAUR, Generalitat de Catalunya" (2014SGR1357) and "Departament de Salut", Government of Catalonia, Spain. We wish to thank all persons who kindly participated in this research. Part of the DNA extractions and genotyping was performed at the Spanish National Genotyping Centre (CEGEN-Barcelona).
11. Harmen H.M.Draisma is supported by an EMGO+ Fellowship as part of the Mental Health research program of the EMGO Institute for Health and Care Research.
12. Sabine Spijker was partially funded by the Center for Medical Systems Biology (CMSB).
13. Sander Goffen was financially supported by grant 91113022 from the Netherlands Organization for Health Research and Development (ZonMW).
14. Marta Ribasés is a recipient of a Miguel de Servet contract from the "Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación", Spain.
15. Barbara Franke's research is supported by grants from the Netherlands Organisation for Scientific Research (NWO; grants 433-09-229 and 016-130-669), from the European Union 7th Framework (grant agreements 278948 (TACTICS), 602450 (IMAGEMEND), and 602805 (Aggressotype)) and Horizon 2020 (grant agreements 643051 (MiND) and 667302 (CoCA)) research programmes.
16. A.B. Smit was partially funded by the Dutch Neuro-Bsik Mouse Pharma Phenomics consortium (grant BSIK 03053 from SenterNovem).

This work was partly carried out on the Dutch national e-infrastructure with the support of SURF Foundation.

Conflict of Interest

J.K.B. has been in the past 3 years a consultant to / member of advisory board of / and/ or speaker for Janssen Cilag BV, Eli Lilly, Shire, Lundbeck, Roche and Servier. He is not an employee of any of these companies, and not a stock shareholder of any of these companies. He has no other financial or material support, including expert testimony, patents, royalties.

J.J.S.K. has been a speaker for Eli Lilly, Janssen and Shire until 2012, and received unrestricted research grants in 2010 from Janssen and Shire.

J.A.R.Q. was on the speakers' bureau and/or acted as consultant for Eli-Lilly, Janssen-Cilag, Novartis, Shire, Lundbeck, Ferrer and Rubió in the last 3 years. He also received travel awards (air tickets + hotel) for taking part in psychiatric meetings from Janssen-Cilag, Rubió, Shire and Eli-Lilly. The ADHD Program chaired by him received unrestricted educational and research support from the following pharmaceutical companies in the last 3 years: Eli-Lilly, Janssen- Cilag, Shire, Rovi and Rubió.

B.F. received educational speaking fees from Shire and Medice.

A.A.V., A.J.G., H.H.M.D., M.K., D.V., S.S., T.E.G., J.J.H., P.J.vdM., V.M. K, R.P., I.M.N., B.W.J.H.P., I.O.F, A.dB., C.M.vD., P.J.H., L.A.K., M.H, M.K., C.M.M., K.G.O., S.H.V., C.S.M., M.R., C.A.H., N.A., A.B.S., D.I.B. report no conflict of interest

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Supplementary Methods

GCTA

Genome-wide Complex Trait Analysis (GCTA; cnsgenomics.com/software/gcta/#Overview) was run in the two largest cohorts included in the meta-analysis, the NTR and NESDA (n=3656 and n=1841 unrelated subjects). A genetic relationship matrix (GRM) among all individuals each data set was estimated, based on SNPs with high imputation quality (RSQR≥0.80). SNPs significantly associated (one-sided test) with genotyping platform were excluded with $P<0.00001$ threshold. In total 6,325,240 SNPs remained in the analysis. The GRM was subsequently used to estimate the proportion of phenotypic variance explained by additive genetic variance, including sex and age, and additionally four principal components (PCs) for population stratification as covariates to adjust for possible population stratification effects Bivariate GCTA was run on the ADHD-index of the CAARS and the ASR ADHD data in the NTR cohort, to assess their genetic correlation.

Exome sequencing

Exomes of 1,336 individuals from the ERF population were sequenced "in-house" at the Center for Biomics of the Cell Biology department of the Erasmus MC, The Netherlands, using the Agilent version V4 capture kit on an Illumina Hiseq2000 sequencer under the TruSeq Version 3 protocol(Amin et al., 2017). Sequence reads were aligned to the human genome build 19 (hg19) in the BWA (bio-bwa.sourceforge.net/) and the NARWHAL (trac.nbic.nl/narwhal/) pipelines. The aligned reads were processed further using the IndelRealigner, MarkDuplicates and TableRecalibration tools from the Genome Analysis Toolkit (GATK; software.broadinstitute.org/gatk/) and Picard (http://picard.sourceforge.net). Genetic variants were called using the Unified Genotyper tool of the GATK. About 1.4 million Single Nucleotide Variants (SNVs) were called and, after removing the low quality variants (QUAL<150) out of Hardy–Weinberg equilibrium and low

call rate, we retrieved 543,954 SNVs in 1,327 individuals. The ADHD index was available for 587 individuals. For prediction of the functionality of the variants, annotations were performed in the SeattleSeq database (snp.gs.washington.edu/SeattleSeqAnnotation131). Detection of rare variants in the ERF study was done for those genes with SNPs with $P<1x10^{-5}$ in the GWAS meta-analysis.

Meta-analysis

Meta-analysis was conducted in METAL (www.sph.umich.edu/csg/abecasis/metal/index.html) by the *P*-value-based method, given the intrinsic variability between the quantitative traits used (see **Supplementary Methods**). Poorly imputed SNPs (RSQR<0.60 or INFO<0.60) and SNPs with low MAF (MAF<1%) were excluded, resulting in a total number of between 5.6 M and 13.4 M SNPs across cohorts available for meta-analysis, and only SNPs overlapping across all cohorts were considered. A list of independently associated SNPs and loci was obtained by clumping the results in PLINK 1.9 (https://www.cog-genomics.org/plink2). The script was downloaded from https://github.com/perslab/gwas-snps-loci, with default settings were used and computed first independent SNPs, that means SNPs below a p-value threshold of $P<1x10^{-05}$ and with low linkage disequilibrium estimates ($r^2<0.1$) to a more significantly associated SNP within a 500-kb window. Second, independent loci were computed, that is loci containing all SNPs correlated at $r^2>0.5$ with any other associated SNP. Associated loci closer to 250 kb to each other were merged. Meta-analysis was performed including and excluding ADHD cases from the IMpACT-NL and VHIR studies.

Linkage disequilibrium score regression (LDSR) analysis

LD scores for HapMap3 SNPs, calculated based on 378 phased European-ancestry individuals from the 1000 Genomes Project, were used in the analyses to obtain the genetic correlations by a two-step procedure in LDSR analysis (https://github.com/bulik/ldsc) for the SAGA ADHD symptom scores and the PGC+iPSYCH meta-analysis (LD scores available on https://github.com/bulik/ldsc). For the PGC+iPSYCH meta-analysis, only markers with an imputation INFO score≥0.9 were included in the analyses. Additionally, only markers overlapping with HapMap3 SNPs were considered and insertions, deletions and strand-ambiguous SNPs were removed. An unconstrained regression was run to estimate regression intercepts for each phenotype. Standard errors were estimated using a block jackknife procedure and used to calculate *P*-values.

Test of heterogeneity for genetic correlations

The test of the equality of the genetic correlations of 0.653 (SE=0.114; PGC+iPsych with 23and Me) and 0.541 (SE=0.447; PGC+iPSYCH with SAGA) was conducted using the Q test of heterogeneity (Hedges, L. V., & Olkin, O. (1985). Statistical methods for meta-analysis. Orlando, FL: Academic Press). To conduct this test, we required the correlation between the estimates of these correlations. We denote this unknown correlation r (rho) to avoid confusion with the actual genetic correlations. Lacking the value of r, we carried out the Q test for a range of correlations (from -0.95 to +0.95). We consistently failed to reject the hypothesis that the correlations (0.653 and 0.541) are equal. The steps involved in this procedure were the following. 1) Fisher z-transformation of the correlations; 2) transformation of the standard errors by means the delta method; 3) the Q test for hypothetical values of r ranging from -0.95 to 0.95 (steps of 0.05). Regardless of the correlation, the Q statistic (centrally $chi^2(1)$ distributed under the null-hypothesis), was consistently insignificant ($P=0.05$), which allowed us to conclude that the correlations, .653 and .541, are equal. The Q test is usually applied to independent correlations (i.e., by design r=0). To check our implementation of the Q-test for dependent correlations, we compared it to the likelihood ratio test of the equality of

correlations, which can be conducted in any structural equation modeling program. The likelihood ratio test and the Q-test were identical.

Functional Analysis

Follow-up functional analyses were performed on the locus containing the best association *P*-value. This locus contains *STXBP5-AS1*, representing a putative long noncoding RNA, predicted to be expressed in several species. Noncoding RNAs at the *STXBP5-AS1* locus are predicted to be expressed in *Pan paniscus* (see RefSeq XR_156214.1, supported by similarity to 11 expressed sequence tags (ESTs)), *Pongo abelii* (XR_152649.1, 8 ESTs), *Nomascus leucogenys* (XR_178064.1, 9 ESTs), *Papio anubis* (XR_160671.1, 6 ESTs), *Chlorocebus sabaeus* (LOC103240196), and *Mus musculus* (LOC102633974, 3 transcripts).

The 3'-splice donor site of the corresponding exon 1 (of the *STXBP5-AS1* locus) is conserved only in man, chimpanzee, and gorilla, whereas the other exons and splice donor sites show conservation also in lower primates (**Supplementary Table 5**), and sometimes in rodents (**Supplementary Fig. 2**).

A reporter plasmid containing the region of *STXBP5-AS1* and *STXBP5* was constructed by inserting exon 1 of human STXBP5 cDNA (ENST00000367481) into pIRES-EGFP using NheI and BstXI. This resulted in an in-frame fusion of amino acid 1–50 with the fluorescent marker EGFP. A control construct was constructed by inserting exon 1 of an irrelevant housekeeping gene, human HPRT1 cDNA (NM_000194), into pmCherry-N1-AgeI using NheI and AgeI. For expression of the long noncoding RNA, a 2,519 bp cDNA encoding *STXBP5-AS1*-003 (ENST00000427394) was cloned into the NheI and NotI sites of pIRES-EGFP, thereby deleting EGFP from the vector. All plasmids use a CMV promoter to drive eukaryotic expression. HEK293 cells were transfected by standard calcium phosphate precipitation. Three days after transfection, cells were fixed with paraformaldehyde, mounted in Mowiol and imaged by epifluorescence with a 40x oil immersion objective and an Orca-Flash4 digital camera (Hamamatsu, Japan). EGFP and mCherry fluorescence intensities were calculated for >900 cells from 40 images and 4 coverslips per experimental condition, using ImageJ software (http://imagej.nih.gov/ij/index.html). To test whether the STXBP5-EGFP/HPRT1-mCherry fluorescence ratio differed significantly in presence or absence of STXBP5-AS1, a Student's t-test was used.

Mouse models

RNA from prefrontal cortex of adult male mice from the inbred strain C57/Bl6J (The Jackson Laboratories; Bar Harbor, Maine, USA; n=7) and recombinant inbred strains BXD29 (The Jackson Laboratories; n=8) and BXD68 (Oak Ridge National Laboratory; Oak Ridge, Tennessee, USA; n=7), bred in the facility of the Neuro-Bsik consortium of the VU University (Amsterdam, Netherlands) and used for behavioral analysis(Loos et al., 2014)[2], was extracted as previously described. Samples were DNase-I treated according to the manufacturer's instructions (20 U/µg RNA; Boehringer, Germany). RNA concentration was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA), and the integrity of RNA was checked by gel electrophoresis (1%-TBE-agarose gel). Random-primed (25 pmol; Eurofins MWG Operon, Germany) cDNA synthesis was performed on individual RNA samples (starting from ~1 µg total RNA). Primers for PCR and real-time quantitative PCR (qPCR) were generated using Primer3.0, and were targeted against exon 2 and 3 of mouse *Stxbp5* (5'-GCCTTTGATCCCGTTCAGAA-3'; 5'-ACGACCAAAGAGCCTTAAAGC-3'), and for the putative mouse *Stxbp5-AS* against the conserved sequence in the mouse genome (5'-10151485GCTGGAGAAATGTCACTGGG10151504-3'; 5'-10151446GTCAGCCTGAAACACTCTTTAGA10151424-3'), corresponding to exon 6 of human *STXBP5-AS1* (**Supplementary Fig. 2**). Real-time qPCR reactions (8 µL; ABI PRISM 7700) were performed in 96-well format plates with transcript-specific primers (300 nM) on cDNA corresponding to ~20 ng RNA (Spijker et al. 2004) and SYBR Green reagents (Applied Biosystems, USA). Expression levels of two housekeeping genes (GAPDH, β-actin) were measured as reference. Amplification efficiencies of primer sets (Eurofins MWG Operon, Germany) were tested by qPCR. Only primers with proper amplification efficiency were used. Cycle threshold (Ct) values were used to calculate the relative level of gene expression, where Ct value is the fractional cycle number at which the fluorescent signal of a reaction passes the threshold (reaching intensity above background). Expression was denoted using normalized Ct values on a $\log_2$-scale. Let normalized Ct-values be denoted by $Ctnorm_x$ (where *x* represents *Stxbp5* or *Stxbp5-AS* expression, *y* represents the geometric mean of Ct-values of the housekeeping genes, and *i* represents a given sample), $Ctnorm_{xi}$ then is given by $Ctnorm_{xi}=Ct_{xi}-Ct_{yi}$. As a bigger Ct-value correlates with a lower expression level, for practical purposes, $Ctnorm_{xi}$ values were converted into $conCtnorm_{xi}$ values, calculated as $conCtnorm_{xi}=-Ctnorm_{xi}+15$. Due to this conversion, the final positive value of *Ct* is positively correlated with relative gene expression level, which makes the visualization simpler. Relative gene expression levels were expressed as conCt*norm*-values±SEM. Thresholds for significance of correlation was set at a *P*-value of $5x10^{-2}/6=0.0083$ (correlations of 2 transcripts were tested for three parameters).

Supplementary Table 1. Quality control, filtering, pre-imputation, and imputation algorithms used.

Project Name	Chip Used	(Pre Imputation) SNPs removed for:			Imputation reference set	Phasing/ Imputation software	Imputation Score Threshold	Association software
		HWE P z< 1e-6	MAF < 0.01	call rate < 95%				
NTR	Various±	For a detailed description see(Nivard et al., 2014)			1000G v3(Abecasis et al., 2012)	Mach/minimac	0.8<INFO<1.1	PLINK v. 1.07
NESDA	Various±	For a detailed description see (Nivard et al., 2014)			1000Gv3(Abecasis et al., 2012)	Mach/minimac	0.8<INFO<1.1	PLINK v. 1.07
ERF	Various±	For a detailed description see(Lubke et al., 2014)			1000G v3(Abecasis et al., 2012)	Mach/minimac	RSQR≥0.6	ProABEL/GenABEL
NeuroIMAGE	Perlegen 600K	278	144511*		1000G v3(Abecasis et al., 2012)	Mach/minimac	RSQR≥0.6	MACH2QTL
BIG	Affymetrix 6.0	12421	136914	63821	1000G v3(Abecasis et al., 2012)	Mach/minimac	RSQR≥0.6	MACH2QTL
NBS	Illumina OmniExpress	45519***			1000G v3(Abecasis et al., 2012) and GoNL4	IMPUTE2	INFO≥0.6	SNPTEST 2.4.1
TRAILS	Illumina Human CytoSNP 12 V2	1005**	31238	3539	1000G v3(Abecasis et al., 2012)	IMPUTE2	INFO≥0.6	SNPTEST
IMpACT-NL	PsychArray24v1-1	81	225454	48191 (<99%)	1000G v3(Abecasis et al., 2012)	Mach/minimac	INFO≥0.6	Mach2Qtl V1.1.2
VHIR	Illumina HumanOmni1-Quad	31	3284	0	1000G v3(Abecasis et al., 2012)	Mach/minimac	RSQR≥0.6	MACH2QTL

*SNPs selected under the following three conditions: 0.01<MAF<0.05 and call rate ≤99%; 0.05 MAF<0.10 and call rate≤97%; and 0.10≥MAF and call rate≤95%, as described in the original publication(Neale et al., 2010); ±NTR and NESDA used a merged set comprising imputed data for samples that were genotyped on different GWA chips. These cohorts used HWE *P*<1E-5 rather than <1E-6 as a QC criterion. ERF also used a merged set comprising imputed data for samples that were genotyped on different GWA chips. **HWE exclusion criteria was based on a *P*-value <10E-4. Checks for relatedness were carried out in those samples that aimed to include unrelated individuals. Genotype data were imputed using the 1000 Genome (1000G) Phase I version 3 (build 37, hg19) reference panel of Caucasian samples in all cohorts. Post-imputation QC removed SNPs with imputation scores (RSQR/INFO) ≤0.6 and MAF<1%. *** Pre-imputation QC used the following thresholds: MAF>1%, HWE>10-4, SNP yield>95%, call rate person > 95%.

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Supplementary Table 2. Number of SNPs tested after imputation and the lambda values per sample.

Project Name	Total Number of SNPs tested	Lambda
NTR	7544891	1.027
NESDA	7242045	1.020
ERF	5670464	1.000
NeuroIMAGE	13473377	0.960
BIG	13472705	0.974
NBS	8522817	1.006
TRAILS	13914043	1.009
IMpACT-NL	8969779	1.011
VHIR	13472568	0.987
Total*	2804754	----

*Total number of SNPs overlapping across all cohorts

Supplementary Table 3. Most strongly associated SNPs (*P*-value<1x10^{-5}) coming from the meta-analysis of nine cohorts from the SAGA consortium in physical position order (hg19).

MarkerName	A1	A2	FreqA1	*P*-value	Direction	HetPVal
6:147409235	**a**	**g**	**0.962**	**3.03x10^{-07}**	**-+++-++++**	**1.29x10^{-02}**
6:147412691	t	c	0.0367	3.45x10^{-07}	++--+----	1.00x10^{-02}
6:147414032	a	g	0.0367	3.58x10^{-07}	++--+----	8.68x10^{-03}
6:147409448	c	*g*	*0.037*	*3.62x10^{-07}*	++--+----	*1.36x10^{-02}*
6:147419417	t	c	0.0364	3.77x10^{-07}	++--+----	8.37x10^{-03}
6:147414186	a	g	0.0368	3.78x10^{-07}	++--+----	8.94x10^{-03}
6:147415636	t	c	0.0369	3.93x10^{-07}	++--+----	8.12x10^{-03}
6:147416176	a	t	0.9631	3.94x10^{-07}	--++-++++	7.86x10^{-03}
6:147416455	t	c	0.0369	3.97x10^{-07}	++--+----	7.77x10^{-03}
6:147421172	a	g	0.0359	4.03x10^{-07}	++--+----	8.13x10^{-03}
22:31035876[¶]	t	c	0.0328	7.33x10^{-07}	---++----	5.50x10^{-01}
6:147377486	t	c	0.0577	8.24x10^{-07}	-+-------	1.42x10^{-01}
6:147383534	a	g	0.942	9.90x10^{-07}	+++++++++	1.60x10^{-01}
6:147384838	t	c	0.0577	1.31x10^{-06}	-+-------	1.33x10^{-01}
6:147385722	t	c	0.0578	1.40x10^{-06}	-+-------	1.36x10^{-01}
6:147387912	a	c	0.0575	1.42x10^{-06}	-+-------	1.32x10^{-01}
6:147386687	t	c	0.0578	1.48x10^{-06}	-+-------	1.26x10^{-01}
6:147389443	c	g	0.9426	1.48x10^{-06}	+++++++++	1.33x10^{-01}
6:147391455	t	g	0.0574	1.58x10^{-06}	-+-------	1.60x10^{-01}
6:147392316	t	c	0.9427	1.82x10^{-06}	+++++++++	1.77x10^{-01}
6:147391341	a	g	0.0568	1.83x10^{-06}	-+--+----	1.21x10^{-01}
2:6510305	**t**	**g**	**0.6703**	**2.15x10^{-06}**	**+++++++++**	**4.47x10^{-01}**

Supplementary Table 3. Continued.

MarkerName	A1	A2	FreqA1	*P*-value	Direction	Het*P*Val
2:6536733	a	g	0.3469	3.41x10^{-06}	-------+-	7.02x10^{-01}
6:147409180	a	g	0.0348	4.62x10^{-06}	++--+----	1.89x10^{-02}
6:147408046	a	c	0.0348	4.66x10^{-06}	++--+----	1.93x10^{-02}
1:83491910	**t**	**c**	**0.5041**	**4.75x10^{-06}**	**---+---+-**	**4.27x10^{-01}**
6:147368934	t	c	0.0796	5.04x10^{-06}	-+--+----	1.38x10^{-01}
4:120042409	**t**	**c**	**0.0165**	**5.77x10^{-06}**	**++-+++++-**	**8.65x10^{-03}**
1:83493185	a	t	0.5035	5.77x10^{-06}	---+---+-	4.32x10^{-01}
1:83491393	c	g	0.5045	6.00x10^{-06}	---+---+-	4.55x10^{-01}
8:3723378	**a**	**g**	**0.0771**	**6.00x10^{-06}**	**+++++++++**	**9.06x10^{-01}**
2:6486352	a	g	0.3345	6.24x10^{-06}	--+------	4.10x10^{-01}
13:69920315	**c**	**g**	**0.1259**	**7.19x10^{-06}**	**-----+-+-**	**2.75x10^{-01}**
6:147390776	t	c	0.9385	7.23x10^{-06}	+-++++++	1.09x10^{-01}
4:120041135	a	g	0.9827	7.45x10^{-06}	--+-----+	1.48x10^{-02}
22:31030104	t	c	0.0205	7.78x10^{-06}	+-+------	3.69x10^{-01}
1:68455306	**a**	**g**	**0.534**	**7.88x10^{-06}**	**--+------**	**8.25x10^{-01}**
4:120042399	t	g	0.9827	7.90x10^{-06}	--+-----+	1.51x10^{-02}
4:120041857	a	c	0.9827	8.02x10^{-06}	--+-----+	1.51x10^{-02}
4:120041779	t	c	0.0173	8.05x10^{-06}	++-+++++-	1.51x10^{-02}
4:120041792	a	t	0.9827	8.06x10^{-06}	--+-----+	1.51x10^{-02}
4:120041495	a	g	0.9827	8.19x10^{-06}	--+-----+	1.48x10^{-02}
8:3723485	a	g	0.9193	8.43x10^{-06}	---------	9.22x10^{-01}
13:69938915	a	g	0.1257	8.44x10^{-06}	-----+-+-	2.27x10^{-01}
13:69930527	t	c	0.8739	8.81x10^{-06}	+++++-+-+	2.34x10^{-01}
13:69909473	a	t	0.8732	9.56x10^{-06}	+++++-+-+	4.70x10^{-01}
8:91190297	**t**	**c**	**0.7029**	**9.61x10^{-06}**	**+++++++++**	**8.64x10^{-01}**
2:6485104	t	c	0.334	9.79x10^{-06}	--+------	3.60x10^{-01}
4:120040823	a	g	0.0173	9.82x10^{-06}	++-+++++-	8.42x10^{-03}
8:3726432	a	t	0.9225	9.95x10^{-06}	---------	9.53x10^{-01}
11:45132165	a	c	0.542	1.11x10^{-05}	++-++++++	3.85x10^{-01}
11:45130956	a	g	0.5418	1.13x10^{-05}	++-++++++	3.64x10^{-01}
3:71549174*	c	g	0.9417	1.15x10^{-05}	---------	8.42x10^{-01}
6:147385308	a	g	0.0796	1.17x10^{-05}	-+--+----	9.39x10^{-02}

¶Cohort order: BIG, NBS, NeuroIMAGE, VHIR, ERF, NESDA, NTR, TRAILS, IMpACT-NL; Highlighted SNPs in **BOLD** we those selected by the clumping analysis; SNP in *italic* is the best associated SNP in the SAG-EAGLE Meta-Analysis; ¶This marker was NOT selected for clumping analysis since there were no other SNPs in LD with it.

Supplementary Table 4. Association results for most strongly associated (clumped) SNPs (*P*-value<1x10^{-6}) coming from the meta-analysis of seven cohorts from the SAGA consortium without including patients in physical position order (hg19).

SNP Name	CHR	POS±	*P*-value	Tested/Non-Tested Allele	Frequency Tested Allele	Gene(s) in Locus
rs1930272*	**1**	**83491910**	**1.90x10^{-6}**	**T/C**	**0.504**	***LOC107985037***
rs13315424	3	16575568	3.37 x10^{-6}	A/G	0.236	Upstream *LINC00690*
rs10027388*	**4**	**120041135**	**5.22 x10^{-6}**	**A/G**	**0.983**	***LOC102723967; LOC105377395***
rs12661753*	**6**	**147409235**	**1.48 x10^{-6}**	**A/G**	**0.962**	***STXBP5-AS1***
rs13274695*	**8**	**3723378**	**8.43 x10^{-6}**	**A/G**	**0.761**	***CSMD1; LOC105377790***
rs7188379	16	5639034	9.39 x10^{-6}	T/C	0.044	*RBFOX1*
rs11914089	22	31035876	5.35x10^{-7}	T/C	0.033	*SLC35E4*

±position based on the GRCh37.p13 build; *SNP or gene also found in the complete analysis.

Supplementary Table 5. Gene-wide association *P*-values for common and rare variants present in the top loci from the SAGA meta-analysis of ADHD Symptom Total Score.

		MAGMA				SKAT	
Gene ID	**EntrezID**	**Full SAGA**		**SAGA+EAGLE**		**ERF**	
		P	**Markers**	***P***	**Markers**	***P***	**Markers**
GNG12-AS1±	100289178	*0.008*	567	0.064	336	N/A	N/A
LINC01247±	101929390	**0.001**	147	0.089	139	N/A	N/A
STXBP5-AS1±	729178	**0.002**	607	**0.001**	526	N/A	N/A
STXBP5¶	134957	*0.010*	131	0.120	121	*0.079*	18
CSMD1	64478	0.909	8389	0.799	6816	0.250	187
CALB1	793	*0.016*	97	0.252	54	0.103	9
LINC00534±	100874052	**0.0003**	279	*0.052*	261	N/A	N/A

Significance threshold=0.05/18 as STXBP5¶ was selected based on the physical proximity to STXBP5-AS1. Genes with significant (**Bold**) or suggestive (*italic*; 0.0028>*P*<0.05) gene-wide association p-values in either analysis are shown; SKAT analysis of rare variants were done in the ERF cohort only; ±GNG12-AS1, LINC01247, STXBP5-AS1, LINC00534 had no SNV available in the exome sequence data from ERF; N/A=Not Available.

Supplementary Table 6. Best associated loci from the PGC+iPSYCH ADHD GWASMA and their association in the SAGA GWASMA.

PGC + iPSYCH ADHD GWASMA											SAGA full		SAGA no patients	
CHR	**BP**	**Index variant**	**genes**	**A1**	**A2**	**A1 Freq**	**OR**	***P*-value**	**Proxy**	**LD to index (r2)**	**Zscore**	***P*-value**	**Zscore**	***P*-value**
1	44184192	rs11420276	ST3GAL3, KDM4A, KDM4A-AS1, PTPRF, SLC6A9, ARTN, DPH2, ATP6V0B, B4GALT2, CCDC24, IPO13	G	GT	0.696	1.113	$2.14x10^{-13}$	rs12410444	0.9755	0.593	0.553	0.863	0.388
1	96602440	rs1222063	Intergenic	A	G	0.328	1.101	$3.07x10^{-8}$	rs1222046	0.4697	0.829	0.407	0.669	0.504
2	215181889	rs9677504	SPAG16	A	G	0.109	1.124	$1.39x10^{-8}$	N/A	N/A	0.619	0.536	0.474	0.636
3	20669071	rs4858241	Intergenic	T	G	0.622	1.082	$1.74x10^{-8}$	N/A	N/A	1.919	*0.055*	1.749	*0.080*
4	31151456	rs28411770	PCDH7, LINC02497	T	C	0.651	1.090	$1.15x10^{-8}$	rs7674790	0.7825	0.588	0.557	0.646	0.519
5	87854395	rs4916723	LINC00461, MIR9-2, LINC02060, TMEM161B-AS1	A	C	0.573	0.926	$1.58x10^{-8}$	rs324886	0.7760	-1.323	0.186	-0.938	0.348
7	114086133	rs5886709	FOXP2, MIR3666	G	GTC	0.463	1.079	$1.66x10^{-8}$	rs7795397	0.9412	0.774	0.439	0.923	0.356
8	34352610	rs74760947	LINC01288	A	G	0.957	0.835	$1.35x10^{-8}$	rs6990255	1.000	1.427	0.154	1.192	0.233
10	106747354	rs11591402	SORCS3	A	T	0.224	0.911	$1.34x10^{-8}$	rs11192262	0.9705	-1.88	*0.060*	-1.788	*0.074*
12	89760744	rs1427829	DUSP6, POC1B	A	G	0.434	1.083	$1.82x10^{-9}$	N/A	N/A	1.607	0.108	1.475	0.140
15	47754018	rs281324	SEMA6D	T	C	0.531	0.928	$2.68x10^{-8}$	N/A	N/A	-1.522	0.128	-1.662	*0.096*
16	72578131	rs212178	LINC01572	A	G	0.883	0.891	$7.68x10^{-9}$	rs7191673	0.6087	-0.052	0.959	0.021	0.983

Replication is tested for the index variant from the ADHD GWAS, or for a proxy variant when the index variant is not present in the SAGA GWASMA. Effect estimates are either Z-scores or odds ratio (OR) with reference to allele 1 (A1); N/A = Not Available

Supplementary Table 7. Conservation and mapping of genomic sequences in primates with similarity to exons encoding human *STXBP5-AS1-003* (Ensembl accession# ENST00000427394)**.**

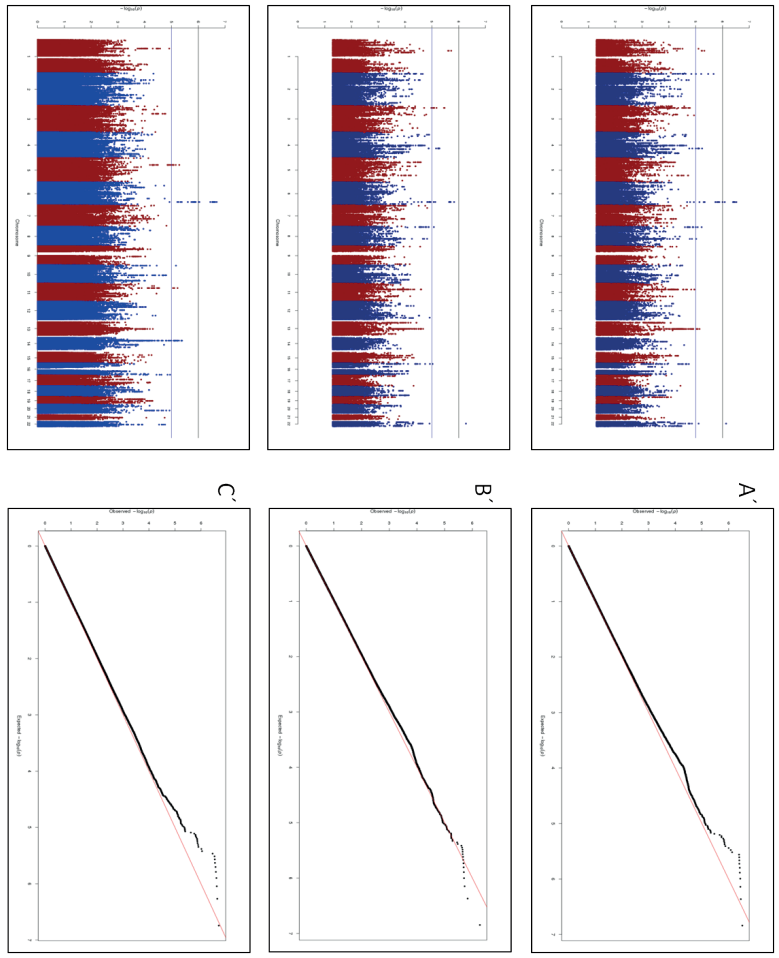
		man	**chimp**	**gorilla**	**orangutan**	**rhesus**	**gibbon**	**baboon**	**green monkey**
	Accession#	NC_00006.12	NC_006473.3	NC_018430.1	NC_012597.1	NC_007861.1	NC_019818.1	NC_018155.1	NC_023654.1
	Chr (ori)	6 (rev)	6 (rev)	6 (rev)	6 (rev)	4 (fw)	3 (rev)	6 (rev)	13 (fw)
exon 1	start	147,204,540	148,746,056	148,138,372	150,276,706	116,144,170	134,657,279	115,462,492	24,559,068
	end	147,204,413	148,745,929	148,138,245	150,276,579	116,144,296	134,657,152	115,462,618	24,559,194
	Identity (%)	100%	97%	100%	98% *)	94% *)	95% *)	94% *)	95% *)
exon 2	start	147,173,948	148,714,870	148,107,674	150,246,589	116,174,395	134,626,697	115,493,195	24,590,355
	end	147,173,815	148,714,737	148,107,541	150,246,456	116,174,528	134,626,564	115,493,242	24,590,488
	Identity (%)	100%	100%	99%	99%	98%	99%	98%	97%
exon 3	start	147,173,647	148,714,569	148,107,373	150,246,288	116,174,701	134,626,400	115,493,415	24,590,661
	end	147,173,593	148,714,515	148,107,319	150,246,234	116,174,750	134,626,346	115,493,464	24,590,710
	Identity (%)	100%	100%	96%	95%	89%	91%	89%	89%
exon 4	start	147,119,477	148,663,138	148,052,574	150,187,224	n.a.	134,572,468	115,547,532	24,647,996
	end	147,119,417	148,663,078	148,052,514	150,187,164	n.a.	134,572,408	115,547,590	24,648,054
	Identity (%)	100%	97%	95%	98%	n.a.	95%	95%	93% *)
exon 5	start	147,074,847	148,615,713	148,006,861	150,141,904	116,277,899	134,523,819	115,593,815	24,694,772
	end	147,074,805	148,615,671	148,006,819	150,141,862	116,277,941	134,523,779	115,593,857	24,694,814
	Identity (%)	100%	100%	98%	98%	100%	95% *)	100%	100%
exon 6	start	147,001,543	148,542,584	147,933,656	150,065,612	116,354,242	134,448,636	115,669,713	24,775,799
	end	147,001,441	148,542,482	147,933,554	150,065,510	116,354,344	134,448,534	115,669,815	24,775,901
	Identity (%)	100%	99%	98%	97%	98%	98%	98%	98%

Supplementary Table 7. Continued

		man	chimp	gorilla	orangutan	rhesus	gibbon	baboon	green monkey
	Accession#	NC_00006.12	NC_006473.3	NC_018430.1	NC_012597.1	NC_007861.1	NC_019818.1	NC_018155.1	NC_023654.1
	Chr (ori)	6 (rev)	6 (rev)	6 (rev)	6 (rev)	4 (fw)	3 (rev)	6 (rev)	13 (fw)
exon 7	start	146,972,070	148,513,404	147,900,318	150,036,460	116,385,520	134,413,998	115,700,560	24,807,498
	end	146,972,045	148,513,379	147,900,293	150,036,435	116,385,545	134,413,973	115,700,585	24,807,523
	Identity (%)	100%	96%	96%	96%	96%	96%	96%	96%
exon 8	start	146,860,743	148,400,637	147,784,816	149,922,827	116,503,950	134,299,158	115,821,252	24,931,511
	end	146,860,618	148,400,512	147,784,691	149,922,702	116,504,075	134,299,036	115,821,374	24,931,633
	Identity (%)	100%	98%	98%	97%	93%	98%	93%	93%
exon 9	start	146,851,331	148,391,230	147,775,399	149,913,146	116,513,227	134,289,776	115,830,888	24,941,536
	end	146,850,825	148,390,724	147,774,893	149,912,642	116,513,739	134,289,270	115,831,400	24,942,048
	Identity (%)	100%	99%	99%	95%	92%	95%	91% *)	92%
exon 10	start	146,844,712	148,384,610	147,768,792	149,896,587	116,519,887	134,283,224	115,837,512	24,948,152
	end	146,844,319	148,384,217	147,768,400	149,896,192	116,520,283	134,282,828	115,837,908	24,948,548
	Identity (%)	100%	97%	97%	94%	90%	93%	90%	90%
exon 11	start	146,842,809	148,382,714	147,766,886	149,894,690	116,521,772	134,281,561	115,839,396	24,950,147
	end	146,842,566	148,382,471	147,766,643	149,894,446	116,522,029	134,281,316	115,839,652	24,950,401
	Identity (%)	100%	99%	98%	94%	88%	95%	88%	89%

Percentages indicate nucleotide identity; asterisk indicates loss of splice donor site conservation; nucleotide number of exon start/end refer to the sequence accession# indicated in the top row. Full species names are (left to right): *Homo sapiens*; *Pan troglodytes*; *Gorilla gorilla, Pongo abelii; Macaca mulatta, Nomascus leucogenys*, *Papio anubis* and *Chlorocebus sabaeus*. Sequence alignments of exon 6 are provided in **Supplementary Figure 2**.

Supplementary Figures



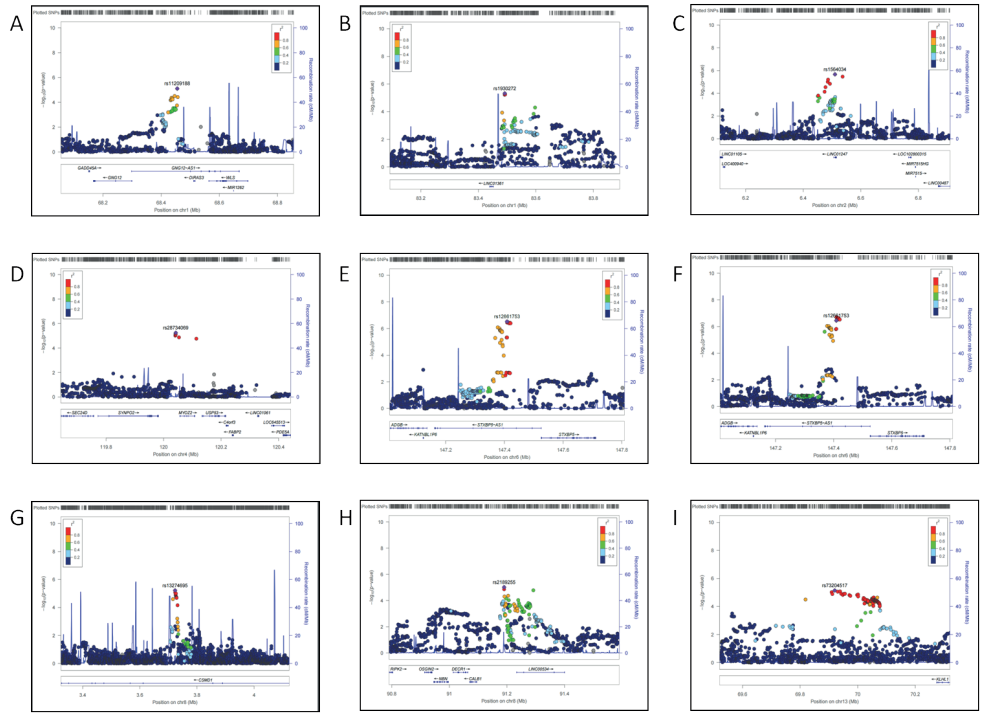
Supplementary Figure 1. Manhattan (**A**) and QQ plot (**A'**)of the ADHD Symptom Total Score meta-analysis from the complete SAGA consortium. Manhattan (**B**) and QQ plot (**B'**) of the ADHD Symptom Total Score meta-analysis from the SAGA consortium without patient cohorts. Manhattan (**C**) and QQ plot (**C'**) of the ADHD Symptom Total Score meta-analysis from the SAGA & EAGLE consortia.

STXBP5-AS1: Exon 6

```
man          agATGCCACTGTTGCTGGAGAAATGTCACTGCTAGAGAACAGAAGACTATTATT
chimp        agATGCCACTGTTGCTGGAGAAATGTCACTGCTAGAGAATAGAAGACTATTATT
gorilla      agATGCCACTGTTGCTGGAGAAATGTCACTGCTAGAGAATAGAAGACTATTATT
orangutan    agATGCCACTGTTGCTGCAGAAATGTCACTGCTAGAGAATAGAAGACTATTATT
rhesus       agATGCCACTGTTGCTGGAGAAATGTCACTGCTAGAGAATAGAAGACTATTATT
gibbon       agATGCCACTGTTGCTGGAGAAATGTCACTGCTAGAGAATAGAAGACTATTATT
baboon       agATGCCACTGTTGCTGGAGAAATGTCACTGCTAGAGAATAGAAGACTATTATT
gr.monkey    agATGCCACTGTTGCTGGAGAAATGTCACTGCTAGAGAATAGAAGACTATTATT
mus          agATGTTGCTGTT**GCTGGAGAAATGTCACTGGG**AAAGATAA-AGGACCATTAT**T**
rat          agATGTTACTGTTGCTGGAGAAATGTCACTATGAAAGATAA-AGGACCATTATT

man          CCAAAGGGTATTTGAGACTGACTGAATCAGGTCTGGAACATTATTGAAATGgt
chimp        CCAAAGGGTATTTGAGACTGACTGAATCAGGTCTGGAACATTATTGAAATGgt
gorilla      CCAAAGGGTATTTGAGACTGACTGAATCAGGTTTGGAACATTATTGAAATGgt
orangutan    CCAAAAGGTATTTGAGACTGACTGAATCAGGTCTGGAACATTATTGAAATGgt
rhesus       CCAAAGGGTATTTGAGACTGACTGAATCAGGTCTGGAATATTATTGAAATGgt
gibbon       CCAAAGGGCATTTGAGACTGACTGAATCAGGTCTGGAACATTATTGAAATGgt
baboon       CCAAAGGGTATTTGAGACTGACTGAATCAGGTCTGGAATATTATTGAAATGgt
gr.monkey    CCAAAGGGTATTTGAGACTGACTGAATCAGGTCTGGAATATTATTGAAATGgt
mus          **CTAAAGAGTGTTTCAGGCTGAC**TGAATCAGGTCTGAAACGTTATTGAAATGgg
rat          CTAAAGAGTGTTTCAGGCTGACTGAATCAGGTCTGAAACCTTATTGAAATGgt
```

Supplementary Figure 2. ClustalW2 alignment of primate and rodent genomic sequences sharing similarity with human *STXBP5-AS1*. Splice acceptor and (possible) donor sites are indicated in lowercase font. See **Supplementary Table 5** for exon start/end positions and sequence accession numbers. The locations of mouse real-time PCR primers, as used in **Figure 3**, are indicated in bold.



Supplementary Figure 3. Locus Zoom plots for SNPs from the SAGA Meta-analysis associated with Total ADHD Symptom Scores. SNP rs11209188 (**A**). SNP rs1930272 (**B**). SNP rs1564034 (**C**). SNP rs28734069 (**D**). SNP rs12661753 (FULL SAGA GWASMA) (**E**).SNP rs12661753 (SAGA-EAGLE GWASMA) (**F**). SNP rs13274695 (**G**). SNP rs2189255 (**H**). SNP rs73204517 (**I**).

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CHAPTER 4

Intellectual Disability-related genes increase ADHD risk and locomotor activity in *Drosophila*

In revision as:

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*authors contributed equally

**shared final responsibility

Abstract

Attention-Deficit/Hyperactivity Disorder (ADHD) is a common, highly heritable neuropsychiatric disorder. ADHD often occurs co-morbid with Intellectual Disability (ID), and shared molecular genetic influences have been suggested. This study aimed to identify novel ADHD genes by investigating whether genes carrying rare mutations linked to ID contribute to ADHD risk through common genetic variants. Validation and characterization of candidates were performed using *Drosophila melanogaster*. Common genetic variants in a diagnostic gene panel of 396 autosomal ID genes were tested for association with ADHD risk, through gene-set and gene-wide analyses, using ADHD meta-analytic data of the Psychiatric Genomics Consortium (n=19,210) for discovery and iPSYCH ADHD data for replication (n=37,076). The significant genes were functionally validated and characterized in *Drosophila* by assessing locomotor activity and sleep upon knockdown of those genes in brain circuits. The ID gene-set was significantly associated with ADHD risk in the discovery and replication data-sets. The three genes most consistently associated were *MEF2C*, *ST3GAL3*, and *TRAPPC9*. Performing functional characterization of the two evolutionary conserved genes in *Drosophila melanogaster*, we found their knockdown in monoaminergic (*dMEF2*) and circadian neurons (*dTRAPPC9*) to result in increased locomotor activity and reduced sleep, concordant with the human phenotype. This study reveals that a large set of ID-related genes contributes to ADHD risk through effects of common alleles. Utilizing this continuity, we identified *TRAPPC9, MEF2C,* and *ST3GAL3* as novel ADHD candidate genes. Characterization in *Drosophila* suggests that *TRAPPC9* and *MEF2C* contribute to ADHD-related behavior through distinct neuronal substrates.

Keywords: Intellectual Disability, ADHD, genetics, *Drosophila melanogaster*, *MEF2C*, *TRAPPC9*

Introduction

Attention-Deficit/Hyperactivity Disorder (ADHD) is a common and highly heritable neurodevelopmental disorder (heritability 70–80%) (Faraone et al., 2015). ADHD is clinically characterized by two core symptom domains: inattention and hyperactivity/impulsivity, which can occur individually or combined (Faraone et al., 2015). The prevalence of ADHD is 5.3% in childhood and 2.5–4.9% in adulthood (Faraone et al., 2015). Despite the high heritability, identification of ADHD risk genes has been difficult, mainly due to ADHD's complex genetic architecture (Faraone et al., 2015). Genetic variants that occur frequently in the population and have generally small individual effects on disease risk are thought to underlie the disorder in most patients, and first genome-wide significant findings for ADHD have been identified only recently (Demontis et al., 2017).

Intellectual Disability (ID) refers to a highly heterogeneous group of childhood-onset disorders characterized by below-average intellectual functioning (IQ<70) and significant limitations in adaptive functioning, which covers many everyday social and practical skills (van Bokhoven, 2011). ID has an estimated prevalence of 2–3% in the population; severe handicaps have a population-prevalence of 0.3–0.5% (Perou et al., 2013). ID is thought to often have monogenic origins, but many different genes and types of mutations are implicated (van Bokhoven, 2011). ADHD is a common comorbid disorder in children with ID (Baker et al., 2010). Studies of children with mild and borderline ID have identified ADHD in 8–39% of the cases (Baker et al., 2010). A recent study using the Swedish birth registry data showed that nearly all of this comorbidity can be attributed to genetic factors (Faraone et al., 2017). Based on such phenotypic and genetic overlap, it has been hypothesized that ID and ADHD, and neurodevelopmental disorders more broadly, have an overlapping genetic etiology (Faraone et al., 2017).

Here, we evaluated the genetic overlap between ID and ADHD in an attempt to identify novel ADHD candidate genes. We investigated, whether genes affected by rare mutations in ID patients also contribute to ADHD risk through common genetic variation. For this, we used the latest data freeze from the Psychiatric Genomics Consortium (PGC; n=19,210) for discovery and the iPSYCH sample (n=37,076) for replication. To provide functional evidence for the newly identified ADHD candidates, we used *Drosophila melanogaster*. This model provided opportunities to characterize the neuronal circuits involved: The role of dopaminergic neurotransmission is well-established in ADHD (Faraone et al., 2015). In addition, circadian rhythm circuits have been implicated, as ADHD often goes together with sleep disturbances, and abnormal circadian rhythms of melatonin secretion have been observed in children and adult patients with ADHD (Baird et al., 2012). Moreover, disrupting the activity of the circadian clock gene *PER1* in both mice and zebrafish revealed ADHD-like symptoms (Huang et al., 2015). We therefore set out to investigate potential dopaminergic and circadian rhythm components of the identified phenotypes in *Drosophila*. Dissecting

the role of neuronal circuits can help to pinpoint the neurotransmitter systems contributing to ADHD as a first step towards an individualization of treatment. Upon downregulation of gene expression pan-neuronally and in the individual circuits, we assessed locomotor activity and sleep as behavioral read-outs, which we have earlier established to be relevant for ADHD (van der Voet et al., 2016).

Material and methods

Ethics statement

The current study used summary statistics of GWAS meta-analyses (GWAS-MA) that had been approved by the local ethics committees and had the required informed consents, as described in the earlier publications (Demontis et al., 2017; Holmans, 2013).

Cohorts

The Psychiatric Genomics Consortium (PGC) ADHD GWAS meta-analysis (GWAS-MA) data, which were used at the discovery stage in this study, were available as autosome-wide summary statistics, including single nucleotide polymorphism (SNP) data with corresponding P-values and odds ratios. Data were based on nine studies including 5,621 cases and 13,589 controls. Samples were of Caucasian or Han Chinese origin and contained patients meeting ADHD-diagnostic criteria according to the DSM-IV (**Supplementary Table 1**). Detailed procedures of DNA isolation, whole-genome genotyping, and imputation have been described previously (Neale et al., 2010). Shortly, genome-wide data were obtained from different genotyping arrays (**Supplementary Table 1**) and was imputed using 1000 Genomes data as a reference panel (v3 phase1 in NCBI build 37 (hg19) coordinates) for autosomal SNPs. Meta-analytic data were processed through a stringent quality control pipeline applied at the PGC (Neale et al., 2010).

The gene-set association was replicated in an independent cohort from the Lundbeck Foundation Initiative for Integrative Psychiatric Research (iPSYCH) - Statens Serum Institut (SSI) – Broad ADHD working group (n=37,076) (Demontis et al., 2017).

A meta-analysis of the two data-sets described above (20,183 cases and 35,191 controls) has recently been published as part of the ADHD Working Group of the PGC and the ADHD iPSYCH-SSI-Broad collaboration (Demontis et al., 2017) (https://www.med.unc.edu/pgc/results-and-downloads). This meta-analytic data-set was used by us to perform a gene-based look-up of three genes of interest, using MAGMA software, as described below. Detailed quality control and imputation parameters have been described in the original publication (Demontis et al., 2017). In short, summary data only included markers with a quality (INFO score) >0.8, minor allele frequency (MAF) >0.01, and supported by an effective sample size greater than 70% (8,047,420 markers) (Demontis et al., 2017).

ID gene selection

For the selection of the ID gene-set, we used the publicly available 'Intellectual Disability Gene Panel' of the Radboudumc department of Human Genetics' Genome Diagnostics division (downloaded from https://issuu.com/radboudumc/docs/ngs-intellectual_disability_panel_1?e=28355229/50899368 on March 27th, 2014). This gene panel listed 490 candidate genes for ID (shown in **Supplementary Table 2**), based on findings of *de novo* mutations in patients with ID visiting the Radboudumc and collaborating institutes and on literature/public databases. This list forms the basis for diagnostic testing using exome sequencing at Radboudumc.

Gene-based and gene-set analysis

Genome-wide summary statistics of ADHD (PGC and iPSYCH ADHD GWAS-MA) used as input for gene-based analyses. We used two software packages to test whether the ID gene-set was associated with ADHD risk. Firstly, the Hybrid set-based test (HYST) of the Knowledge-based mining system for Genome-wide Genetic studies (KGG) version 3.5 software (Li et al., 2010) was used for association testing (**Supplementary Methods**). Secondly, the Multi-marker Analysis of GenoMic Annotation (MAGMA) software version 1.02 (de Leeuw et al., 2015) was used (**Supplementary Methods**). The analyses were carried out in two steps. In step 1, the combined effect of the SNPs in (the vicinity of) all ID genes was analyzed. Post hoc, in step 2, the potential effects of the individual genes were investigated, by reviewing their gene-based test-statistics. Genes were considered gene-wide significant if they reached the Bonferroni correction threshold adjusted for the number of genes tested (P<0.000128).

Functional characterization of *MEF2C* and *TRAPPC9* in *Drosophila melanogaster*

Strains and cultivation

Drosophila orthologues were retrieved from NCBI protein DELTA-BLAST and ENSEMBL gene-tree (Boratyn et al., 2012; Yates et al., 2016). The *Drosophila* orthologues of *MEF2C* (*Mef2*) and *TRAPPC9* (*brun*) were targeted for knockdown through RNA interference (RNAi) using the *UAS-GAL4* system. Conditional knockdown was achieved using tissue-specific promoters driving GAL4-expression. Several neuronal populations were targeted: *nSyb-GAL4* (*yw* UAS-Dcr-2 hs*(X); ; *nSyb-GAL4*) (Dietzl et al., 2007) targets all neurons (pan-neuronal driver), *tim-GAL4* (; *tim*-GAL4, *UAS-Dcr-2*/CyO;) (Pandey et al., 2013) targets mostly circadian rhythm neurons, and *ple-GAL4* (w*; *UAS-Dcr-2*; *ple-GAL4*) (Dietzl et al., 2007) targets tyrosine hydroxylase-expressing (dopaminergic) neurons. A copy of *UAS-Dcr-2* was incorporated to improve knockdown efficiency (Dietzl et al., 2007). The driver stocks were crossed with *UAS*-RNAi lines obtained from the Vienna *Drosophila* Resource Center: v12482 (w^{1118}; *UAS-dTRAPPC9*^{RNAi};), v15549 (w^{1118}; ; *UAS-dMef2*$^{RNAi-1}$), and v15550 (w^{1118}; ; *UAS-dMef2*$^{RNAi-2}$). Stock

v60000 (w^{1118}) served as the genetic background control. All flies were maintained on standard corn meal food at 28°C with 60% relative humidity in 12-hour light:dark cycle.

Locomotor activity monitoring

Locomotor activity of individual 3–5 days old male flies was recorded with the *Drosophila* Activity Monitoring system (Trikinetics, Waltham, USA). The flies were collected with the aid of CO_2 and allowed to recover for 24 hours. The activity count was recorded for four days at 28°C and 60% RH in 12-hour light:dark cycle, followed by two days in constant darkness. The activity data was collected every 30-seconds and analyzed in 1-minute bins. Activity and sleep were analyzed with pySolo software (Gilestro and Cirelli, 2009), defining sleep as ≥5 minutes of inactivity. The average daily activity and sleep were then plotted in 10- and 30-minute bins, respectively. pySolo software was modified to analyze the total activity and sleep between 180–540 min Zeitgeber Time (ZT) for the relative day and 900–1260 min ZT for the relative night to capture periods of stable activity and sleep, as described previously (van der Voet et al., 2016). Data of individual flies from at least two independent experiments were pooled and t-tests were performed with Welch's correction, when variances were unequal. Results were considered significant, if they reached the Bonferroni correction threshold adjusted for the number of drivers tested (P<0.0167). To compare the relative day and night activity, the delta (Δ) activity and sleep between knockdown and genetic background control were calculated (Δ^{day}=knockdown^{day}–control^{day} and Δ^{night}=knockdown^{night}–control^{night}). All statistical analyses were performed with GraphPad Software (San Diego, CA).

Results

Association of ID gene-set with ADHD risk

To select candidate genes for the ID gene-set, we used the publicly available 'Intellectual Disability Gene Panel'. Genes were included based on findings of *de novo* mutations in patients with ID visiting the Radboudumc and collaborating institutes and on literature/public databases (n=490; **Supplementary Table 2**). The set of ID genes was tested for association with ADHD using two different software algorithms in a discovery-replication design. For discovery, we used the PGC ADHD genome-wide association study meta-analysis (GWAS-MA) data (n=19,210) and the KGG software; the HYST test revealed that the ID gene-set as a whole was significantly associated with ADHD risk (P_{KGG}=0.0001; n_{genes}=387). To assess the robustness of our findings, we tested the association of the ID gene-set with ADHD in the PGC data using the MAGMA software. The results also showed a significant association of the ID gene-set with ADHD risk in the self-contained test ($P_{self\text{-}contained}$=0.0412; n_{genes}=392), but not in the competitive test ($P_{competitive}$=0.9522). As an independent replication, we tested

the gene-set association in the iPSYCH cohort (n=37,076) using MAGMA; the results robustly replicated the significance in the self-contained test ($P_{self\text{-}contained}$=1.2429x10^{-13}; n_{genes}=393). The competitive test was negative again ($P_{competitive}$=0.5306).

To identify the major contributors (i.e. most significantly associated individual genes) to the observed association for further validation, we performed individual gene-wide testing within the gene-set using the PGC data. The most consistent findings across algorithms were for the Myocyte Enhancer Factor 2C gene (*MEF2C*; P_{KGG}=1.3x10^{-5} and P_{MAGMA}=1.497x10^{-4}; **Fig. 1A**), the Trafficking Protein Particle Complex 9 gene (*TRAPPC9*; P_{KGG}=7.81x10^{-7} and P_{MAGMA}=0.0035; **Fig. 1B**), and the ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 3 gene (*ST3GAL3*; P_{KGG}=6.18x10^{-5} and P_{MAGMA}=6.808x10^{-4}; **Fig. 1C**). Gene-based p-values for all genes in both KGG and MAGMA analyses can be found in **Supplementary Table 3**. A look-up in the recently published combined PGC+iPSYCH GWAS-MA (Demontis et al., 2017) revealed genome-wide significant results for gene-wide analysis of *ST3GAL3* and *MEF2C*, and nominal significance for *TRAPPC9* ($P_{ST3GAL3}$=4.6406x10^{-13}, P_{MEF2C}=2.671x10^{-10}, and $P_{TRAPPC9}$=0.0184).

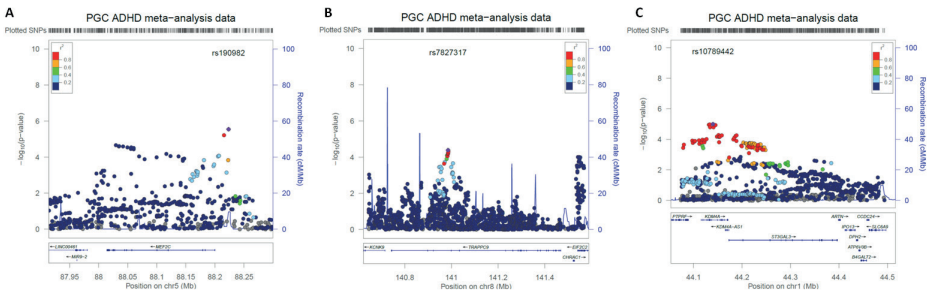

Figure 1: Regional association plots showing association signals for ADHD in the PGC GWAS-MA (n=19,210) for the three most consistently associated genes, including flanking regions of 100 kb. (**A**) *MEF2C* locus with the top-SNP (rs190982) indicated by the purple dot. (**B**) *TRAPPC9* locus with the top-SNP (rs7827317) indicated by the purple dot. (**C**) *ST3GAL3* locus with the top-SNP (rs10789442) indicated by the purple dot. Results are shown as –log (p-value) for genotyped and imputed SNPs. The color of each marker reflects its LD (r^2) with the strongest associated SNP (in purple). The recombination rate is plotted in blue. cM/Mb, centimorgan/megabase. Chr, chromosome.

Functional validation and characterization of *MEF2C* and *TRAPPC9* in *Drosophila*

Next, we investigated the validity of the newly identified ADHD candidate genes by mapping their effects on ADHD-related phenotypes in the fruit fly *Drosophila melanogaster*. We did so by investigating different neuronal circuits individually in addition to pan-neuronal knockdown of the genes. This allowed us to characterize the different pathways through which individual ADHD risk genes may act. Secondly, it may reveal phenotypes that might otherwise be masked by opposing actions of different neuronal circuits (Sitaraman et al., 2015). We have earlier established *Drosophila* as a model for ADHD by showing that pan-

neuronal knockdown of ADHD genes caused (dopamine-related) night-specific increased locomotor activity and sleep loss (van der Voet et al., 2016). Two of the three candidates were found conserved in *Drosophila*: the *MEF2* gene-family homolog *Mef2* (further referred to as *dMEF2*) and the *TRAPPC9* homolog *brun* (further referred to as *dTRAPPC9*). *ST3GAL3* is mainly found in vertebrates, and no known orthologue has been identified in *Drosophila*. We investigated locomotor activity and sleep after knocking down the *dMEF2* and *dTRAPPC9* in the whole nervous system, and in dopaminergic or circadian rhythm neurons, specifically. Conditional knockdown was achieved by driving the expression of RNA interference (RNAi) in the neuronal populations of interest (pan-neuronal, dopaminergic, and circadian rhythm) using the binary UAS/Gal4 system. Flies were monitored in 12-hour light:dark scheme, mimicking day and night period. We also investigated behavior in 24-hour constant darkness conditions, given our earlier model that the dopamine-related increased locomotor activity is present in the absence of light (van der Voet et al., 2016). Locomotor activity and sleep were quantified for relative day and relative night periods, reflecting the stable period of activity and sleep, as previously described (van der Voet et al., 2016).

dMEF2 knockdown gives rise to elevated night-time activity and reduced sleep

Pan-neuronal knockdown of *dMEF2* expression caused no changes in activity and sleep during the relative day compared to the genetic background control (**Fig. 2A**), but significantly increased night activity ($P_{activity}$=0.0059) and reduced sleep (P_{sleep}=0.014) (**Fig. 2A**). In constant darkness, the knockdown also showed significantly increased activity ($P_{activity}$=0.0088) and less sleep (P_{sleep}=0.00037) in the relative night period (**Fig. 2B**). The data from individual RNAi lines are visualized in **Supplementary Figure 1**. Knockdown of *dMEF2* in dopaminergic neurons recapitulated the findings from the pan-neuronal knockdown, and showed an even stronger effect. The knockdown showed increased night activity ($P_{activity}=1.8x10^{-15}$) and reduced sleep ($P_{sleep}=5.1x10^{-15}$; **Fig. 2C**). Activity and sleep during the relative day period were not different from the genetic background control (**Fig. 2C**). In constant darkness, increased activity and reduced sleep were observed in both relative day ($P_{activity}=4.5x10^{-7}$, $P_{sleep}=2.6x10^{-17}$) and night ($P_{activity}=1.6x10^{-17}$, $P_{sleep}=9.5x10^{-26}$; **Fig. 2D**). Knockdown in circadian rhythm neurons did not yield flies. Thus, activity and sleep were not monitored. **Supplementary Table 4** and **5** list all tests performed and their results.

dTRAPPC9 knockdown influences activity and sleep only in neuronal subtypes

Pan-neuronal knockdown of *dTRAPPC9* did not result in observable alterations in activity or sleep in either the 12-hour light:dark cycle (**Fig. 3A**) or in constant darkness (**Fig. 3B**). Specific knockdown of *dTRAPPC9* in dopaminergic neurons caused significantly reduced activity and increased day sleep during the relative day ($P_{activity}$ =0.0022; P_{sleep}=0.013), but not in the night (**Fig. 3C**). In constant darkness, relative night activity was increased and sleep was reduced ($P_{activity}$=0.012; P_{sleep}=0.015; **Fig. 3D**). In contrast, knockdown of *dTRAPPC9*

in circadian rhythm neurons resulted in increased night activity and reduced night sleep ($P_{activity}=4.2x10^{-5}$; P_{sleep}=0.00022; **Fig. 3E**). In constant darkness, increased activity and reduced sleep were also present in the relative night ($P_{activity}$=0.00017; P_{sleep}=0.010; **Fig. 3F**). Lists of all tests performed and their results can be found in **Supplementary Table 4** and **5.**

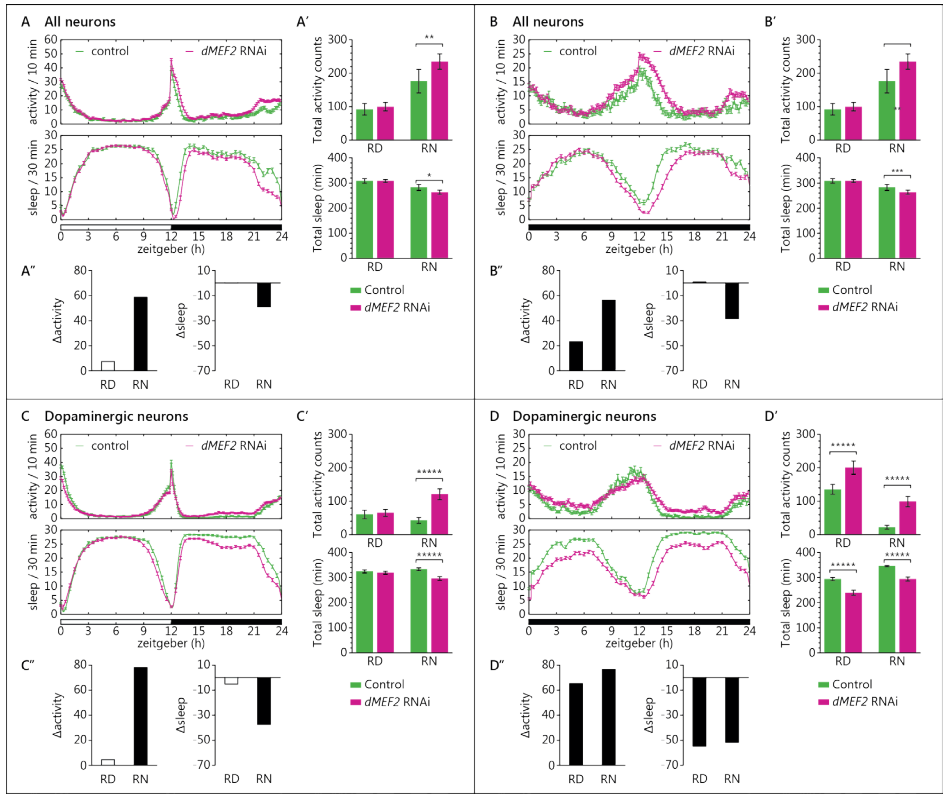

Figure 2: Knockdown of *dMEF2* in the whole nervous system, and in dopaminergic neurons specifically, results in higher activity and reduced sleep in the relative night. **(A, B)** Activity and sleep plot of pan-neuronal *dMEF2* knockdown, **(A)** in 12-hour light:dark cycle and **(B)** in constant darkness. **(A', B')** Quantification of total activity and sleep during the stable period. **(A", B")** $\Delta_{activity}$ and Δ_{sleep}: the findings for 12-hour light:dark cycle and for constant darkness both reveal that the difference between groups is greater in the absence of light. **(C, D)** Activity and sleep plot of dopaminergic neuron *dMEF2* knockdown, **(C)** in 12-hour light:dark cycle and **(D)** in constant darkness. **(C', D')** Quantification of total activity and sleep during the stable period. **(C", D")** $\Delta_{activity}$ and Δ_{sleep}: the findings for 12-hour light:dark cycle and for constant darkness reveal that the difference is greater when light is absent. For the figure, data from two *dMEF2* lines with identical *UAS*-RNAi constructs were combined; the individual data are presented in **Fig. S1**. RD, relative day; RN, relative night. Error bars represent standard error of means (SEM). *P<0.0167 (Bonferroni correction threshold), **P<0.01, ***P<0.001, ****P<0.0001, *****P<0.00001.

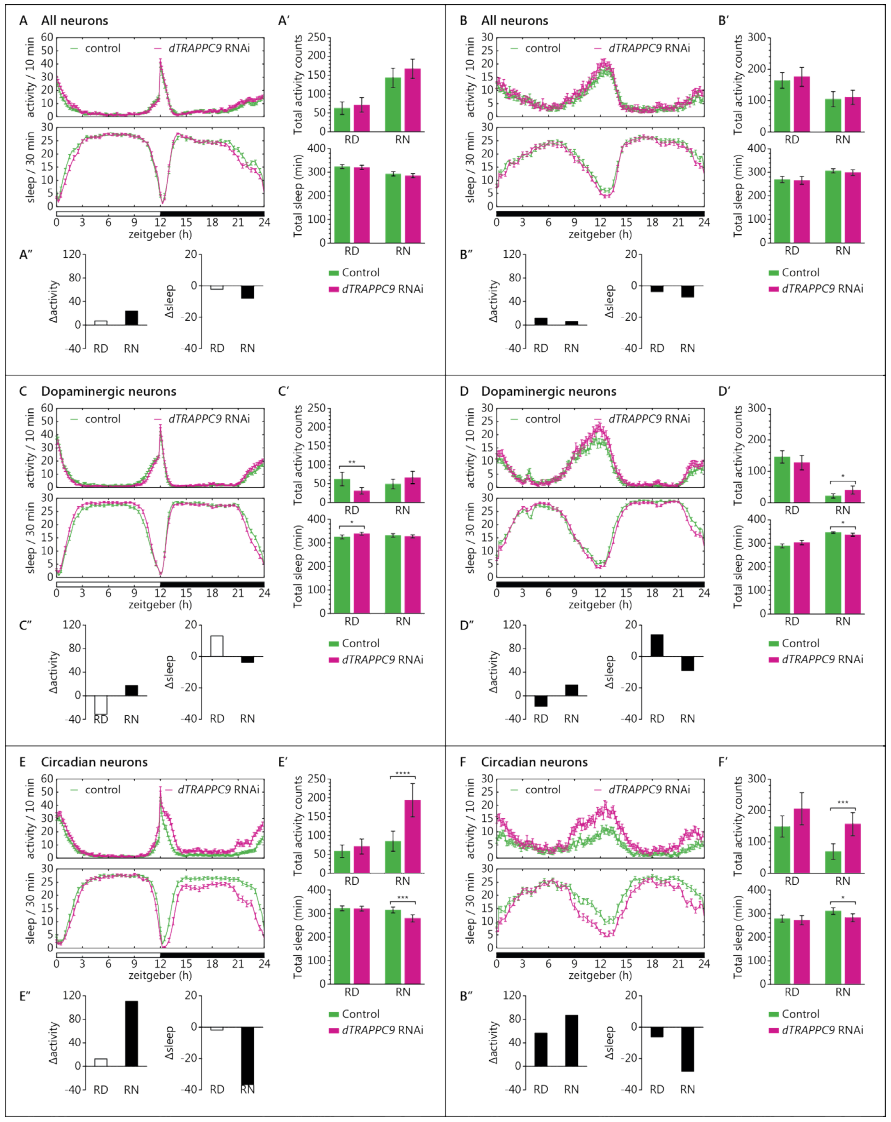

Figure 3: Knockdown of *dTRAPPC9* results in higher activity and reduced sleep, when induced in circadian rhythm neurons, but not in the whole nervous system or dopaminergic neurons. **(A, B)** Activity and sleep plot of pan-neuronal *dTRAPPC9* knockdown, **(A)** in 12-hour light:dark cycle and **(B)** in constant darkness. **(A′, B′)** Quantification of total activity and sleep during the stable period. **(A″, B″)** $\Delta_{activity}$ and Δ_{sleep}. **(C, D)** Activity and sleep plot of dopaminergic neuron *dTRAPPC9* knockdown, **(C)** in 12-hour light:dark cycle and **(D)** in constant darkness. **(C′, D′)** Quantification of total activity and sleep during the stable period. **(C″, D″)** $\Delta_{activity}$ and. **(E, F)** Activity and sleep plot of circadian rhythm neuron *dTRAPPC9* knockdown, **(E)** in 12-hour light:dark cycle and **(F)** in constant darkness. **(E′, F′)** Quantification of total activity and sleep during the stable period. **(E″, F″)** $\Delta_{activity}$ and Δ_{sleep}: the findings for 12-hour light:dark cycle and for constant darkness reveal that the difference is greater when light is absent. RD, relative day; RN, relative night. Error bars represent standard error of means (SEM). *P<0.0167 (Bonferroni correction threshold), **P<0.01, ***P<0.001, ****P<0.0001, *****P<0.00001.

Discussion

In the current study, we used a robust discovery-replication design in the currently largest available, independent data sets to show that genes affected by rare genetic variation in ID patients also contribute to ADHD risk through common genetic variation. In the discovery phase, we also used different algorithms to test gene-set association to further test the robustness of findings. In the KGG HYST test and MAGMA, we found significance in both self-contained tests but not the competitive test. A non-significant competitive p-value in the competitive test should be interpreted as an inability to disentangle the part of the polygenicity attributable to the genes in the gene-set from the polygenicity "remaining" (i.e. not captured by the set) on the rest of the genome. In combination with a significance in the self-contained test, it should not be interpreted as no effect of the selected gene-set on the outcome. Our replication in the larger, independent data-set makes this point convincingly. Even more convincing is the fact that two of the three novel ADHD candidate genes that we identified, *MEF2C* and *ST3GAL3*, are among the genome-wide significant findings in the recently published ADHD GWAS-MA (Demontis et al., 2017).

Interestingly, our study design produced reproducible findings in much smaller sample sizes than those needed to reach genome-wide significance, which makes such overlap studies an attractive source of novel disease genes. While we based our selection of ID genes on a diagnostic gene panel, many more ID genes are currently being discovered through the fast advances in next generation sequencing technology; those surely leave additional ADHD genes to be identified.

Our interdisciplinary approach, combining highly powered statistical analyses in humans with functional analyses in an unconventional, validated *Drosophila* model for ADHD-related behavior (Klein et al., 2015; van der Voet et al., 2016), allowed for a direct validation and further characterization of neuronal substrates involved. None of our three top-genes had been investigated in the context of ADHD before. *MEF2C* encodes a member of the MADS box transcription factor, which binds to the conserved MADS box sequence motif (Janson et al., 2001). *MEF2C* is important for normal neuronal function by regulating neuronal proliferation and dopaminergic neuron differentiation, survival, and synapse development (Adachi et al., 2016; Li et al., 2008). It also plays a role in hippocampal-dependent learning and memory, possibly by controlling the number of excitatory synapses (Barbosa et al., 2008). While both haplo-insufficiency and gene-duplications of *MEF2C* give rise to ID in humans, most severe ID cases are linked to large deletions removing part or all of *MEF2C* and *de novo* point mutations in the gene (Rocha et al., 2016); individuals with duplications of *MEF2C* usually display a milder phenotype, with only mild cognitive impairment (Novara et al., 2013). This is why we chose to model reduced gene-expression in *Drosophila* in this study. Common variants (SNPs) in the *MEF2C* locus have previously been found associated with various cognitive, neuropsychiatric, and neurodegenerative phenotypes, such as intelligence (Sniekers et al.,

2017), schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics, 2014), and Alzheimer's disease (Beecham et al., 2014), indicating pleiotropic effects of this gene on a range of phenotypes. The findings of our study add ADHD to this list and suggest that this is linked to the role of *MEF2C* in neurotransmission mainly through dopaminergic neurons. However, knowing that in *Drosophila dMEF2* expression is important in maintaining normal circadian rhythm (Blanchard et al., 2010; Sivachenko et al., 2013), we cannot yet rule out an additional role of this circuit in the ADHD-related behaviors as our *dMEF2* knockdown did not yield flies.

TRAPPC9 has been implicated in NF-kB activation and is possibly involved in intracellular trafficking. *TRAPPC9* is highly expressed in postmitotic neurons of the cerebral cortex, and MRI analysis of affected patients showed defects in axonal connectivity (Mochida et al., 2009). The *Drosophila TRAPPC9* has been studied for its involvement in meiotic division in *Drosophila* male gametes (Robinett et al., 2009), but a neuronal function has not been described so far. *TRAPPC9*-associated ID is linked to loss of function of the gene (Marangi et al., 2013). Hyperactive behavior has so far been reported in one patient with a *TRAPPC9* mutation (Philippe et al., 2009). Our findings indicate that *TRAPPC9* plays a role in neurodevelopmental disorders and suggest that the gene acts by affecting brain circuits regulating circadian rhythm.

The third ADHD candidate, *ST3GAL3*, is not conserved in *Drosophila*, hence we were not able to study its contribution to ADHD-relevant behavior. The gene encodes a membrane protein (ST3GalIII) that adds sialic acid to the terminal site of glycolipids or glycoproteins. The gene is expressed in a variety of tissues including neurons (Yoo et al., 2015). In mice, *St3gal2* and *St3gal3* are responsible for nearly all the terminal sialyation of brain gangliosides and play an important role in cognition (Yoo et al., 2015). A role in brain development is also likely in humans, as the human brain is particularly enriched in sialic acid-containing glycolipids (i.e. gangliosides) (Wang, 2012). Gangliosides are known to modulate calcium homeostasis and signal transduction in neurons (Wu et al., 2001). Common genetic variants in *ST3GAL3* have also been associated with educational attainment (Okbay et al., 2016). Interestingly, in a recent study of DNA-methylation, sites annotated to *ST3GAL3* were found associated with ADHD symptom trajectories in the population (Walton et al., 2017). The use of alternative animal models, e.g. the zebrafish, is warranted to characterize the neuronal circuits underlying *ST3GAL3*'s effect on ADHD-related behavior further.

In summary, the genetic overlap we observed between ID and ADHD may suggest biological pleiotropy, in which genetic variation severity in an overlapping set of genes is linked to the severity of neurodevelopmental phenotypes. Functional characterization of neuronal substrates involved revealed that the novel ADHD candidate genes may impact disease etiology through different biological pathways.

Acknowledgements

Funding: Part of this work was carried out on the Dutch national e-infrastructure with the support of SURF Foundation. The authors would like to acknowledge grants supporting their work from the Netherlands Organization for Scientific Research (NWO), i.e. the NWO Brain & Cognition Excellence Program (grant 433-09-229), the Veni Innovation Program (grant 91-614-084 to MV), and the Vici Innovation Program (grant 016-130-669 to BF). Additional support is received from the Dutch National Science Agenda for the NWANeurolabNL project (grant 400 17 602), from the European Community's Seventh Framework Programme (FP7/2007 – 2013) under grant agreement n° 602805 (Aggressotype), from the European Community's Horizon 2020 Programme (H2020/2014 – 2020) under grant agreements n° 643051 (MiND), n° 667302 (CoCA), and n° 728018 (Eat2beNICE), by the ECNP Network ADHD across the Lifespan, and from the Lundbeck Foundation (grant numbers R102-A9118 and R155-2014-1724) to iPSYCH. Computational resources for handling and statistical analysis of iPSYCH data on the GenomeDK HPC facility were provided by the Centre for Integrative Sequencing, iSEQ, Aarhus University, Denmark (grant to ADB).The ADHD working group of the Psychiatric Genomics Consortium (PGC) and the iPSYCH-SSI-Broad collaboration ADHD Working Group contributed with independent sets of summary of consortium findings. The data and a complete list of contributing samples and people can be obtained from the PGC download website (https://www.med.unc.edu/pgc/results-and-downloads).

Author contributions

Study conception and supervision: M.K., E.S., A.S., M.vdV., B.F. Obtained funding: A.S., M.vdV., B.F., A.D.B. Provided samples and/or data: D.D, A.D.B. Conducted analyses: M.K., E.S., A.vR., D.D. Contributed to analyses and data interpretation: H.G.B, A.A.-V. Writing group: M.K., E.S., M.vdV., B.F. All authors reviewed and approved the final version of this manuscript.

Conflict of interest

Barbara Franke received educational speaking fees from Shire and Medice. All other authors report no conflicts of interest.

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Supplementary methods

Gene-based and gene-set analysis

We extracted SNPs and association p-values from the PGC ADHD GWAS-MA for the ID gene-set. Since this meta-analysis only covered the autosomes, X-chromosomal genes were excluded, leaving 396 autosomal genes. All SNPs lying within these genes (according to UCSC hg19 position (Kent et al., 2002)), including flanking regions of 100 kb to capture regulatory regions, were extracted. In total, 308,952 SNPs with a MAF ≥0.01 and INFO-score ≥0.8 were considered for further analysis. The gene-based and gene-set association analyses in the iPSYCH data were performed at secured servers in Denmark at the GenomeDK high performance-computing cluster (http://genome.au.dk) by the responsible researcher using the same protocols.

We used two software packages to test whether the ID gene-set was associated with ADHD risk. Firstly, the Hybrid set-based test (HYST) of the Knowledge-based mining system for Genome-wide Genetic studies (KGG) version 3.5 software (Li et al., 2010) was used for association testing. Within this software package, we chose the Hybrid set-based test (HYST) (Li et al., 2012) for association testing. A text file listing all 396 autosomal ID genes and a text file listing all SNPs that were extracted from the PGC ADHD GWAS-MA, were used as input for KGG. Imputed and quality-controlled genome-wide genotyping data of the Brain Imaging Genetics (BIG) cohort (Guadalupe et al., 2014) were used as a reference to define the underlying linkage disequilibrium (LD) structure. The LD upper limit was set to a r^2 of 0.8, while the lower limit was set to 0.2. Next, a gene-based association scan was run with HYST, based on a hybrid test of Gates and a scaled Chi-square test, in which SNPs without LD information were ignored. Gene-set and gene-based p-values were calculated for 388 genes, since eight genes could either not be annotated by the reference file (*ATP1A2, ERCC2, GRIK2, GSS, KIAA1279, MMACHC*, and *TCF4*) or had too few SNPs left after quality control (*B4GALT7*).

Secondly, the Multi-marker Analysis of GenoMic Annotation (MAGMA) software version 1.02 (de Leeuw et al., 2015) was used. First, genome-wide SNP data from a reference panel (1000 Genomes, v3 phase1 (Genomes Project et al., 2010)) were annotated to NCBI Build 37.3 gene locations using a symmetric 100 kb flanking window, and both files were downloaded from http://ctglab.nl/software/magma. Next, the gene annotation file was used to map the genome-wide SNP data to assign the SNPs to the genes and to calculate gene-based p-values for each cohort separately. For the gene-based analyses, single SNP p-values within a gene were transformed into a gene-statistic by taking the mean of the χ2-statistic among the SNPs in each gene. To account for LD, the 1000 Genomes Project European sample was used as a reference to estimate the LD between SNPs within (the vicinity of) the genes (http://ctglab.nl/software/MAGMA/ref_data/g1000_ceu.zip). Gene-wide p-values were converted to z-values reflecting the strength of the association of each gene with the phenotype, with higher z-values corresponding to stronger associations. Subsequently, we tested, whether

all 396 ID-related genes in the gene-set are jointly associated with each of the phenotypes, using an intercept-only linear regression model including a subvector corresponding to the genes in the gene-set. This self-contained analysis evaluated, whether the regression coefficient of this regression was larger than 0, testing whether the gene-set contains any association at all. To test if the genes in the gene-set are more strongly associated with each phenotype than other genes, the regression model was then expanded including all genes outside the gene-set. This competitive test tested, whether the association of a gene-set is different from association of genes outside the gene-set. To account for the potentially confounding factors of gene size and gene density, both gene size and gene density as well as their logarithms were included as covariates in the competitive gene-set analysis. Four genes were not included in the analyses, because they were either missing from the annotation file (*CHKB-CPT1B* and *SOX2-OT*) or contained too few SNPs (*B4GALT7* and *RAB3GAP2*).

The analyses were carried out in two steps. In step 1, the combined effect of the SNPs in (the vicinity of) all ID genes was analyzed. Post hoc, in step 2, the potential effects of the individual genes were investigated, by reviewing their gene-based test-statistics. Genes were considered gene-wide significant if they reached the Bonferroni correction threshold adjusted for the number of genes tested (P<0.000128).

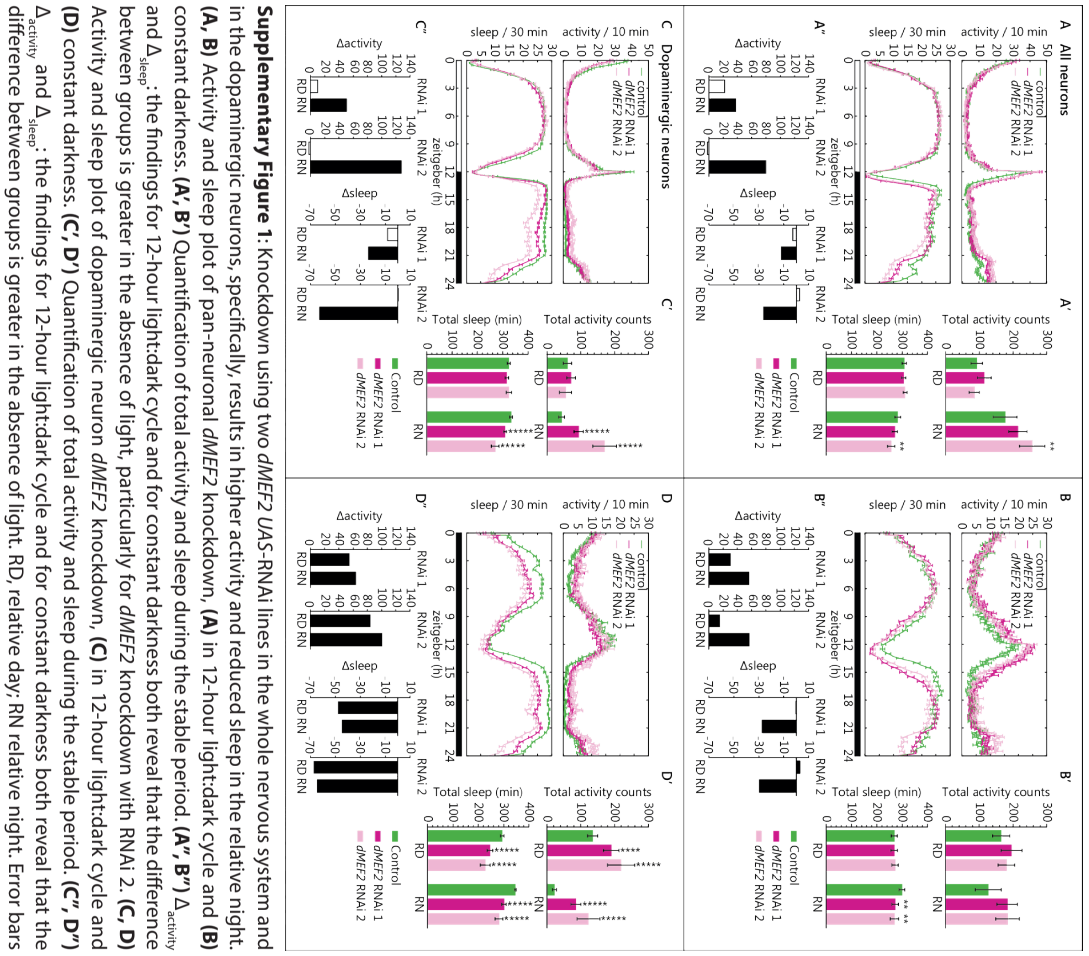
URLs

https://www.med.unc.edu/pgc/results-and-downloads
http://ctglab.nl/software/MAGMA/ref_data/g1000_ceu.zip
http://locuszoom.sph.umich.edu/
https://issuu.com/radboudumc/docs/ngs-intellectual_disability_panel_1?e=28355229/50899368

Data availability

GWAS summary statistics used in the paper are available directly from the web for ADHD GWAS-MA data of the PGC ADHD Working Group and the combined ADHD GWAS-MA data of the PGC ADHD Working Group of the PGC + ADHD iPSYCH-SSI-Broad collaboration (https://www.med.unc.edu/pgc/results-and-downloads). The ID gene-set can be downloaded from https://issuu.com/radboudumc/docs/ngs-intellectual_disability_panel_1?e=28355229/50899368 on March 27th, 2014. Fly strains are available in the Vienna Drosophila Resource Center and Bloomington Drosophila Resource Center as mentioned in the online methods, or upon request. All data needed to evaluate the conclusions in the paper are present in the paper or the supplementary materials.

Supplementary figures



Supplementary Figure 1: Knockdown using two *dMEF2 UAS*-RNAi lines in the whole nervous system and in the dopaminergic neurons, specifically, results in higher activity and reduced sleep in the relative night. **(A, B)** Activity and sleep plot of pan-neuronal *dMEF2* knockdown, **(A)** in 12-hour light:dark cycle and **(B)** constant darkness. **(A', B')** Quantification of total activity and sleep during the stable period. **(A", B")** $\Delta_{activity}$ and Δ_{sleep}: the findings for 12-hour light:dark cycle and for constant darkness both reveal that the difference between groups is greater in the absence of light, particularly for *dMEF2* knockdown with RNAi 2. **(C, D)** Activity and sleep plot of dopaminergic neuron *dMEF2* knockdown, **(C)** in 12-hour light:dark cycle and **(D)** constant darkness. **(C', D')** Quantification of total activity and sleep during the stable period. **(C", D")** $\Delta_{activity}$ and Δ_{sleep}: the findings for 12-hour light:dark cycle and for constant darkness both reveal that the difference between groups is greater in the absence of light. RD, relative day; RN relative night. Error bars represent standard error of means (SEM). *P<0.0167 (Bonferroni correction threshold), **P<0.01, ***P<0.001, ****P<0.0001, *****P<0.00001.

Supplementary tables

Supplementary Table 1. Description of individual PGC ADHD cohorts as included in the PGC ADHD GWAS-MA.

Study name		Study design	Ancestry	Cases (N[a])	Controls (N[a])	Genotyping platform	Reference[b]
Short	Full sample name						
CARD	UK sample	Case/control	Caucasian	727	5,081	Illumina Human660W-Quad BeadChip (ADHD cases) and Illumina Human 1.2M BeadChip (controls)	(Stergiakouli et al., 2012)
CHIN	Chinese sample	Case/control	Han Chinese	1,040	963	Affymetrix 6.0 array	(Yang et al., 2013)
CHOP	Children's Hospital of Philadelphia	Trio/control	European descent	335 trios	2026	Illumina Infinium II HumanHap550 BeadChip	(Elia et al., 2010)
GERM	ADHD patient sample consisted of children and adolescents Aachen, Cologne, Essen, Marburg, Regensburg, and Würzburg	Case/control	Caucasian (all German)	495	1,300	Illumina Human660W-Quadv1(ADHD cases) and Illumina HumanHap550v3 (controls)	(Hinney et al., 2011)
CROS	Toronto University, SickKids project	Trio	Mainly Caucasian	170		Affymetrix 6.0 array	(Lionel et al., 2011)
IMAGE1	Phase I of the International Multisite ADHD Genetics Project	Trio	Western European origin	909 trios		Perlegen Sciences 600 K	(Neale et al., 2008)
IMAGE2	Phase II of IMAGE	Case/control	Predominantly European origin	896	2,455	Affymetrix 5.0 array (ADHD cases) and Affymetrix 6.0 arrays (controls)	(Neale et al., 2010)
PuWMa	Pfizer funded study from the University of California, Los Angeles, Washington University and the Massachusetts General Hospital	Trio	Mainly Caucasian	735 trios		Illumina Human1M or Human1M-Duo BeadChip platform	(Mick et al., 2010)
SPAN	Spanish sample	Case/control	Caucasian	607	584	Illumina HumanOmni1-Quad BeadChip platform	(Sanchez-Mora et al., 2014)
TOTAL				**5,621**	**13,589**		

[a] Based on sample of primary publication [b] Primary publication reporting individual study sample.

Supplementary Table 2. ID gene panel listing 490 candidate genes for ID

Gene	Chr	Omim disease
ADAR	1	Aicardi-Goutieres syndrome 6
ADCK3	1	-
AKT3	1	Megalencephaly-polymicrogyria-polydactyly-hydrocephalus syndrome
ALG6	1	Congenital disorder of glycosylation type Ic
AP4B1	1	Spastic paraplegia 47 autosomal recessive
ARID1A	1	Mental retardation autosomal dominant 14
ASPM	1	Microcephaly 5 primary autosomal recessive
ATP1A2	1	Alternating hemiplegia of childhood
DARS2	1	Leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation
DBT	1	Maple syrup urine disease type II
DHCR24	1	Desmosterolosis
DPYD	1	5-fluorouracil toxicity
FH	1	Fumarase deficiency
FUCA1	1	Fucosidosis
GALE	1	Galactose epimerase deficiency
GATAD2B	1	Mental retardation autosomal dominant 18
GJC2	1	Leukodystrophy hypomyelinating 2
GNPAT	1	Chondrodysplasia punctata rhizomelic type 2
HAX1	1	Neutropenia severe congenital 3 autosomal recessive
MMACHC	1	Methylmalonic aciduria and homocystinuria cblC type
MTR	1	Homocystinuria-megaloblastic anemia cblG complementation type
NDUFS2	1	Mitochondrial complex I deficiency
NLRP3	1	CINCA syndrome
NTRK1	1	Insensitivity to pain congenital with anhidrosis
ORC1	1	Meier-Gorlin syndrome 1
PEX10	1	Peroxisome biogenesis disorder 6A (Zellweger)
PEX11B	1	Peroxisome biogenesis disorder 14B
…	…	...

*The complete **Supplementary Table 2** is available upon request.*

Supplementary Table 3. Results of gene-based association analyses for 396 ID-related genes with ADHD risk in the PGC ADHD GWAS-MA data (n=19,210). Results for both KGG and MAGMA analyses, using a 100 kb flanking region, are shown.

Symbol	EntrezID	Chromosome	KGG		MAGMA	
			# SNPs	P	# SNPs	P
MEF2C	4208	5	699	0.000013	546	0.0001497
ST3GAL3	6487	1	1189	0.0000618	776	0.00068088
BBS7	55212	4	628	0.000347	537	0.0013212
GPR56	9289	16	585	0.00278	533	0.0017071
ASL	435	7	594	0.000773	498	0.0025384
TRAPPC9	83696	8	2241	0.000000781	1979	0.0035331
ARFGEF2	10564	20	995	0.00927	728	0.0040172
PEX26	55670	22	543	0.0248	470	0.0046159
GUSB	2990	7	513	0.0153	431	0.0058534
ALG1	56052	16	321	0.00461	272	0.014585
MAT1A	4143	10	852	0.00515	709	0.017966
LIG4	3981	13	536	0.00445	451	0.019828
ATR	545	3	945	0.0228	591	0.021092
ALG2	85365	9	564	0.053	455	0.022287
ABCC9	10060	12	572	0.036	451	0.023248
RFT1	91869	3	647	0.00377	545	0.0269
NDUFA11	126328	19	203	0.192	179	0.027452
TCF4	6925	18	N/A	N/A	1016	0.027539
CRBN	51185	3	973	0.0185	773	0.028749
KCTD7	154881	7	773	0.0207	655	0.029168
BSCL2	26580	11	242	0.0428	181	0.031856
SMOC1	64093	14	1283	0.0334	1092	0.033258
SLC35C1	55343	11	575	0.0275	499	0.037417
RMND1	55005	6	817	0.117	652	0.041922
NDUFS4	4724	5	1371	0.0271	1009	0.045524
RNASEH2A	10535	19	138	0.0464	123	0.047939
DIP2B	57609	12	928	0.0385	662	0.049517
…	…	…	…	…	…	…

N/A: no gene-based result obtained for this gene.

*The complete **Supplementary Table 3** is available upon request.*

Supplementary Table 4. Summary of results of functional characterization of *dMef2* and *dTRAPPC9* knockdown in *Drosophila* in 12-hour light:dark cycle.

	Number of flies (N)	Total RD activity	P-value	Total RN activity	P-value	Total RD sleep	P-value	Total RN sleep	P-value
Pan-neuronal									
dMef2 control	62	92.63		176.65		308.69		282.78	
dMef2 RNAi	135	100.13	0.50	235.25	0.0059	308.66	1.0	263.72	0.014
dMef2 RNAi 1	68	114.76	0.10	214.30	0.092	306.92	0.64	270.71	0.14
dMef2 RNAi 2	67	85.29	0.52	256.51	0.0026	311.45	0.63	256.62	0.0049
dTRAPPC9 control	66	62.78		143.33		324.20		293.48	
dTRAPPC9 RNAi	68	69.82	0.58	167.14	0.19	321.97	0.70	285.50	0.22
Dopaminergic neurons									
dMef2 control	132	61.09		43.36		324.85		333.36	
dMef2 RNAi	185	65.74	0.57	121.43	$1.8x10^{-15}$*	319.76	0.24	295.92	$5.1x10^{-15}$*
dMef2 RNAi 1	119	71.52	0.25	94.09	$3.5x10^{-09}$*	316.65	0.086	309.90	$5.7x10^{-08}$
dMef2 RNAi 2	66	55.33	0.60	170.72	$8.1x10^{-10}$*	325.37	0.93	270.72	$8.6x10^{-12}$*
dTRAPPC9 control	84	62.54		49.22		325.63		331.70	
dTRAPPC9 RNAi	79	31.06	0.0022*	66.75	0.0931	338.74	0.013*	327.93	0.47
Circadian neurons									
dMef2 control	*Not tested*								
dMef2 RNAi	*Not tested*								
dMef2 RNAi 1	*Not tested*								
dMef2 RNAi 2	*Not tested*								
dTRAPPC9 control	56	58.30		83.22		322.48		316.39	
dTRAPPC9 RNAi	53	71.22	0.19	193.96	4.2x10-05	320.73	0.80	279.80	0.00022

*Welch correction was performed. RD=relative day. RN=relative night.

Supplementary Table 5. Summary of results of functional characterization of *dMef2* and *dTRAPPC9* knockdown in *Drosophila* in constant darkness.

	Number of flies (N)	Total RD activity	P-value	Total RN activity	P-value	Total RD sleep	P-value	Total RN sleep	P-value
Pan-neuronal									
dMef2 control	62	164.99		126.95		269.14		299.56	
dMef2 RNAi	133	188.18	0.17	183.37	0.0088	270.45	0.95	271.17	0.00037*
dMef2 RNAi 1	67	195.66	0.13	183.03	0.023	268.76	0.97	272.25	0.0022
dMef2 RNAi 2	66	180.58	0.39	183.72	0.030	272.16	0.74	270.07	0.0047*
dTRAPPC9 control	66	164.47		104.77		269.70		306.25	
dTRAPPC9 RNAi	66	176.08	0.56	110.72	0.72	269.99	0.72*	299.15	0.35*
Dopaminergic neurons									
dMef2 control	128	135.52		22.63		294.56		346.07	
dMef2 RNAi	173	200.79	$4.5x10^{-07}$*	99.08	$1.6x10^{-17}$*	239.79	$2.6x10^{-17}$*	294.47	$9.5x10^{-26}$*
dMef2 RNAi 1	112	190.39	$8.5x10^{-05}$*	86.06	$5.5x10^{-13}$*	246.57	$1.0x10^{-11}$*	301.43	$8.7x10^{-17}$*
dMef2 RNAi 2	61	219.88	0.00016*	122.98	$1.4x10^{-07}$*	227.34	$8.9x10^{-09}$*	281.69	$2.1x10^{-11}$*
dTRAPPC9 control	83	145.91		21.78		289.57		345.81	
dTRAPPC9 RNAi	78	127.61	0.23	40.35	0.012*	303.54	0.030*	336.82	0.015*
Circadian neurons									
dMef2 control	*Not tested*								
dMef2 RNAi	*Not tested*								
dMef2 RNAi 1	*Not tested*								
dMef2 RNAi 2	*Not tested*								
dTRAPPC9 control	55	149.22		69.96		278.91		311.48	
dTRAPPC9 RNAi	53	206.00	0.067	157.03	0.00017	272.45	0.62	283.35	0.011

*Welch correction was performed. RD=relative day. RN=relative night.

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CHAPTER 5

From man to fly – convergent evidence links *FBXO25* to ADHD and comorbid psychiatric phenotypes

Submitted as:

B. Harich*, **M. Klein***, C. Ockeloen, M. van der Voet, N. de Leeuw, M. Naber, A. Schenck**, B. Franke** (2018). "From man to fly – convergent evidence links *FBXO25* to ADHD and comorbid psychiatric phenotypes".

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Abstract

Mental disorders, including Attention-Deficit/Hyperactivity Disorder (ADHD), have a complex etiology, and the identification of underlying genetic risk factors has been challenging. This study used a multi-step approach to identify and validate a novel risk gene for ADHD and psychiatric comorbidity. In a single family, severely affected by ADHD and comorbid disorders, we applied microarray analysis to detect Copy Number Variants (CNVs) linked to disease. Genes present in the identified CNV were subsequently tested for their association with ADHD in the largest data set currently available (n=55,374); this analysis was based on common genetic variants and used the MAGMA software. Significant findings were taken forward for functional validation using *Drosophila melanogaster* as biological model system, altering gene expression with the GAL4-UAS system and a pan-neuronal driver and subsequently characterizing locomotor activity and sleep as functional read-out. We identified a copy number gain in 8p23.3, which segregated with the psychiatric phenotypes in the family and that was confirmed by quantitative PCR. Common genetic variants in this locus were associated with ADHD, especially the *FBXO25* and *TDRP* genes. Only *FBXO25* is conserved in *Drosophila*. Overexpression of the orthologue in two models consistently led to increased locomotor activity and reduced sleep compared to the genetic background control. Our integrative approach combines ADHD risk gene identification in an individual family with genetic association testing in a large case-control data set and functional validation in a model system to show that *FBXO25* contributes to key features of ADHD and comorbid neuropsychiatric disorders.

Keywords: ADHD, psychiatric comorbidities, *FBXO25*, *TDRP*, *Drosophila melanogaster*

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a neuropsychiatric disorder with a high prevalence, affecting 5–6% of children and 2.5%–4.9% of adults (Faraone et al., 2015). Individuals with this disorder are at high risk of developing co-morbid psychiatric disorders across the entire lifespan(Franke et al., in revision). Twin and adoption studies have shown that ADHD is highly heritable, with heritability estimates of approximately 80% in both children and adults(Faraone et al., 2005; Larsson et al., 2014). ADHD is a heterogeneous disorder with a complex, multifactorial background. The identification of genes affecting the disorder has been challenging (Demontis et al., 2017; Franke et al., 2012; Gizer et al., 2009). In the last decade, genome-wide association studies (GWAS) have been performed with increasing sample sizes, and the first genome-wide significant risk variants for ADHD from a GWAS meta-analysis, all of them having small individual effect sizes, have recently been reported (Demontis et al., 2017). In addition to studies of common genetic variants contributing to ADHD, a second wave of studies has focused on rare variants, which potentially have larger effect sizes. So far, those studies mainly investigated copy-number variations (CNV) and identified rare CNVs in patients with ADHD (Akutagava-Martins et al., 2014; Elia et al., 2010; Elia et al., 2011; Jarick et al., 2014; Lesch et al., 2011; Lesch et al., 2008; Martin et al., 2014; Ramos-Quiroga et al., 2014; Williams et al., 2012; Williams et al., 2010; Yang et al., 2013), currently only few exome sequencing studies are available (Corominas et al., in revision; Demontis et al., 2016; Kim et al., 2017). In addition to the case-control study design, CNVs have been identified in studies using a family-based approach (Lesch et al., 2008). Now that first risk factors for ADHD have been identified from common and rare variant approaches, it becomes clear that at least part of ADHD's comorbidity with other disorders, like major depression and the psychotic disorders, is based on genetic factors (Demontis et al., 2017; Lee et al., 2013; van Hulzen et al., 2017).

If a variant is only seen once in a case-control study of rare variants or in a single family, it is hard to prove the association with a psychiatric phenotype based on statistics. Similarly, in studies of common genetic risk factors for psychiatric phenotypes, it is often difficult to go beyond association and prove causality of a specific gene, as variants tested through GWAS are just anonymous markers. An effective way forward in this is the use of suitable animal models, where one can specifically manipulate the expression of individual genes of interest. We have recently shown that the fruit fly *Drosophila melanogaster* is an appropriate model for ADHD; we could show that the fly exhibits increased locomotor activity, when expression of ADHD candidate genes is altered (van der Voet et al., 2016). Around 75% of human genes have a clear orthologue in the fly (van der Voet et al., 2016). The *Drosophila* model combines a vast genetic toolbox based on decades of research with a large range of quantifiable behaviors (van Alphen and van Swinderen, 2013) and is inexpensive in its use.

In this study, we used a multi-step approach to identify and validate a novel risk gene for ADHD and comorbid psychiatric conditions. In a single family, severely affected by ADHD and comorbid disorders (especially psychosis), we applied genome-wide single nucleotide polymorphism (SNP)-based array analysis to detect rare copy number variants (CNVs) co-segregating with disease. Genes present in the CNV were tested - as a set and individually - for association with ADHD and other relevant psychiatric disorders based on common genetic variants, using the largest internationally available data sets. The significantly associated gene *FBXO25* was taken forward for functional validation in *Drosophila*. We characterized the effects of CG11658 (*dFBXO25)* over-expression using locomotor activity and sleep behavior as functional read-outs.

Methods and materials

Study participants and cohorts

Dutch family

The male index patient had diagnoses of ADHD, anxiety disorder, and multiple complex developmental disorder (MCDD) at the age of four years; he also showed aggressive behavior and suicidal tendencies, had experienced a psychotic episode, and reported somatic abnormalities including gastric reflux and clinodactyly (**Supplementary Methods**). His parents (mother diagnosed with ADHD, father without diagnosis), the mother's brother (psychotic episode, aggressive behavior, alcohol addiction, childhood ADHD symptoms, currently homeless), the mother's sister (learning difficulties), and two of the mother's cousins (ADHD) were also ascertained for genetic testing (see **Figure 1** for the pedigree). For these members of the family, saliva (using Oragene containers; DNA Genotek, Ottawa, Ontario, Canada) or blood was collected. All human material was collected after approval by the local ethic committees, and participants in this study gave written informed consent.

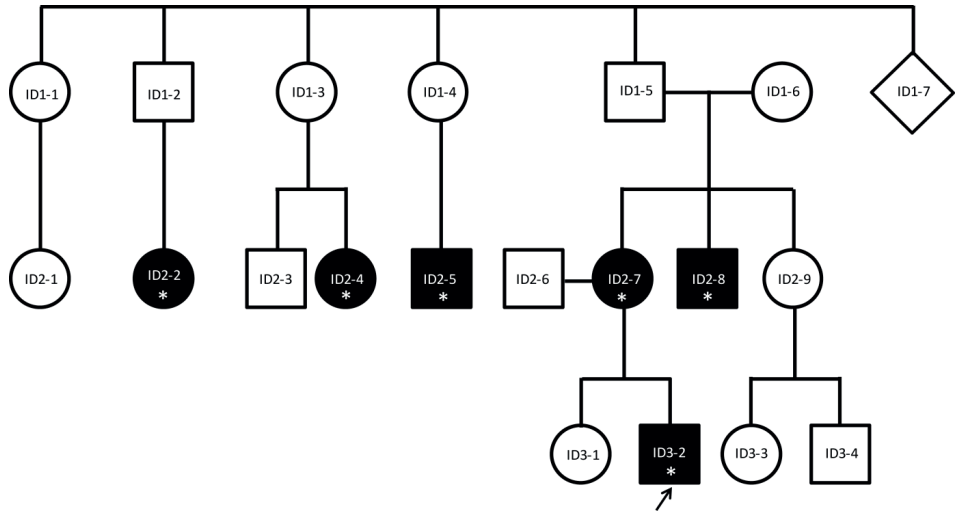

Figure 1: Pedigree of the family under study. Patients with ADHD with or without psychiatric comorbidity are depicted in black (see Supplementary Methods for an exhaustive description of their phenotypes), unaffected family members are shown by white symbols. Grey symbols show family members with learning difficulties. The index patient is marked by an diagonal arrow. An asterisk beneath an individual's code indicates that DNA was used for qPCR analysis.

GWAS meta-analyses data sets for ADHD and schizophrenia

We obtained genome-wide association study meta-analysis (GWAS-MA) results in the form of summary statistics (p-values and odds ratios) from the largest currently available data sets on ADHD and schizophrenia (SCZ). GWAS-MA data on ADHD were obtained from the ADHD Working Group of the Psychiatric Genomics Consortium (PGC) and the ADHD iPSYCH-SSI-Broad collaboration. Data were downloaded from (https://www.med.unc.edu/pgc/results-and-downloads; n=55,374 (Demontis et al., 2017)). GWAS-MA data on schizophrenia were obtained from the SCZ working group of the PGC (n=82,315; https://www.med.unc.edu/pgc/results-and-downloads (Schizophrenia Working Group of the Psychiatric Genomics, 2014)). Only variants with an imputation quality score INFO >0.8 and minor allele frequency (MAF) >0.01 were taken forward for further analyses. Details of inclusion criteria, genotyping, and phenotype characteristics are described in the original publications (Demontis et al., 2017; Schizophrenia Working Group of the Psychiatric Genomics, 2014) and the **Supplementary Methods**.

Copy number analysis in the Dutch family

Genome-wide single nucleotide polymorphism (SNP)-based array analysis was performed on DNA from peripheral blood of the index patient and his parents using the Affymetrix CytoScan HD array platform (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocols. The probe values from 2.6 million markers, consisting of 750,000 SNPs and 1.9 million non-polymorphic probes, were analyzed using the Affymetrix software

package ChAS (Chromosome Analysis Suite) at an average resolution of approximately 20 kb and based on human genome build 37 (hg19). Detection and reporting criteria were used as previously described (Kooper et al., 2014). Follow-up testing by Fluorescence in situ hybridization (FISH) was performed with a subtelomeric 8pter probe (TelVysion 8p (D8S504); Abbott Molecular, Des Plaines, IL, USA).

The observed CNV was subsequently confirmed by real-time, quantitative PCR (qPCR) in the index patient and his mother, and three additional family members were analyzed using this method. Genomic DNA was either isolated from saliva or from EDTA blood samples according to manufacturer's protocol at the department of Human Genetics of the Radboud university medical center, Nijmegen, The Netherlands. PrimeTime® Mini qPCR assay (Integrated DNA Technologies [IDT], Coralville, IA, USA) was used to determine the copy number of *FBXO25* (for details see **Supplementary Methods**). Primer sequences are displayed in **Table S1**. Results were analyzed with the 7500 Software v2.0.6 (Life Technologies) using an automatic threshold (**Supplementary Methods**). Data was visualized using GraphPad prism (version 5.03), and the mean and a 95% confidence interval are shown.

Gene-set and gene-based analyses in the PGC+iPSYCH ADHD and PGC schizophrenia data sets

The cumulative effect of common variants in the set of protein-coding genes altered in the family (as a set and individually) was tested using summary statistics of the ADHD and the SCZ GWAS-MA (**Supplementary Methods** (Demontis et al., 2017; Schizophrenia Working Group of the Psychiatric Genomics, 2014)). Statistical analyses were performed using the Multi-marker Analysis of GenoMic Annotation (MAGMA) software package (version 1.05; http://ctglab.nl/software/magma (de Leeuw et al., 2015)) using the SNP-wide mean model for gene-based association analysis (for detailed description see **Supplementary Methods**). A correction for linkage disequilibrium (LD) was applied, based on the estimates from the 1000 Genomes Phase 1 European ancestry samples (Genomes Project et al., 2010). Protein-coding genes were considered gene-wide significant, if they reached the Bonferroni-corrected threshold adjusted for the number of genes tested (three tests; P<0.0167).

Brain gene expression

We determined the messenger-RNA (mRNA) expression of genes located within the identified CNV. Using the publicly available data set provided by the Human Brain Transcriptome Project (Kang et al., 2011) at http://hbatlas.org, we assessed mRNA expression trajectories in six regions of the developing and adult human brain. Spanning periods from embryonic development to late adulthood, this data set provides genome-wide exon-level transcriptome data generated using the Affymetrix GeneChip Human Exon 1.0 SS Arrays

from over 1,340 tissue samples from both hemispheres of postmortem human brains (n=57) (GTEx Consortium, 2013).

Functional Characterization of *FBXO25* in *Drosophila*

Drosophila stocks and maintenance

Conditional overexpression of CG11658 (two-to-one orthologue of *FBXO25 and FBXO32*) was achieved by using the GAL4-UAS system and the pan-neuronal driver *UAS*-Dcr-2 hs(X); *nSyb*-GAL4. UAS overexpression lines were obtained from the stock centers in Kyoto and Bloomington (FBXO25 overexpression-1: Kyoto stock 203566 and FBXO25 overexpression-2: Bloomington stock 17663). The genetic background of the overexpression lines was outcrossed for eight generations using an isogenic wild-type background (Iso31, gift from A. Sehgal; (Kumar et al., 2012)). Flies were raised on standard medium (cornmeal, sugar, yeast) and maintained on a 12 hours light dark cycle. Crosses with the pan-neuronal driver and the UAS over-expression lines were raised at 28°C.

Locomotor activity and sleep measurement

Locomotor activity and sleep were assessed with the *Drosophila* Activity Monitor system (DAM), which measures locomotor activity by infrared photoelectric barriers (Trikinetics, Waltham, MA, USA). Activity of individual 3–5 day-old male flies was recorded over 4 days on a 12h light:dark cycle and subsequently 2 days in constant darkness. Single adult flies were transferred to monitor tubes, which contained standard food. Activity counts were collected in 30-s bins and analyzed in 1-min bins. Five minutes of inactivity of a fly was defined as sleep. The analysis was performed with the pySolo software package, and activity and sleep in the light-off period (relative night) (between 12–24h) was analyzed. Activity counts represent the amount of infrared detection beam passes of the fly. Statistical analysis was performed with the GraphPad Prism software package 5.03 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). T-tests were performed, adding a Welch correction, when variances of groups were significantly different.

Quantification of relative gene expression

Flies carrying the UAS element driving the overexpression of CG11658 (Bloomington 17663 and Kyoto 203566) and their genetic background control were crossed with *yw*; *UAS*-Dcr-2 hs(X); *nSyb*-GAL4 driver and raised at 28°C. Per condition, three biological replicates of 10 heads of 3-day-old flies were collected and snap frozen. RNA was extracted using the ARCTURUS PicoPure RNA Isolation Kit (Applied Biosystems, Waltham, MA, USA), total RNA concentration was measured with the Nanodrop (Thermo fisher scientific, Waltham, MA, USA). Complementary cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Relative gene expression per sample (3 biological replicates) was quantified in technical triplicates using a powerSYBR Green PCR master mix (Applied

Biosystems, Waltham, MA, USA) on a 7900HT Real-Time PCR Systems (Applied Biosystems). Crossing-point (CP) values of the *CG11658* (dFBXO25) gene were normalized to the reference genes αTub84B and *elF-1A*. For primer sequences see **Table S1**.

Whole-exome sequencing (WES) in Dutch family

Genomic DNA of the index patient and both parents isolated from blood was used for whole exome sequencing (WES) to search for *de novo* mutations using protocols described previously (Neveling et al., 2013). In short, exome capture was performed with the Agilent SureSelect Human All Exon enrichment kit (Agilent Technologies, Santa Clara, California, USA). WES was performed on the Illumina HiSeq platform (BGI, Copenhagen, Denmark). Data were analyzed with Burrows-Wheeler Alignment (BWA) read alignment (Li and Durbin, 2009) and Genome Analysis Toolkit (GATK) variant calling (McKenna et al., 2010) software packages. Variants were annotated using an in-house developed pipeline(Neveling et al., 2013). Prioritization of variants was done by an in-house designed 'variant interface' and manual curation in line with international standards and guidelines for the interpretation of sequence variants (Richards et al., 2015).

Results

8p23.3 microduplication identified in a family with ADHD-affected members

In a family with several members affected with ADHD in the presence or absence of psychiatric comorbidity (**Figure 1**), microarray analysis revealed that the index patient, his mother, and mother's brother carried a copy number gain of approximately 540 kb at the distal end of the short arm of chromosome 8 (arr[hg19] 8p23.3(158,049–541,637)x3), which spanned 590 probes (**Figure 2**). Follow-up testing by FISH revealed that the gain in copy number was the result of a duplication rather than an unbalanced translocation. The microduplication encompassed three protein-coding genes, *ZNF596*, *FBXO25*, and *TDRP*, and the pseudo-gene *RPL23AP53*. The copy number gain was confirmed by qPCR through analysis of *FBXO25* (**Figure S1**). The qPCR analysis showed that duplication of *FBXO25* was absent in one ADHD-affected family member, but was present in the mother's sister, who had learning difficulties (**Figure S1**).

Common variants in the 8p23.3 locus are associated with ADHD

Focusing on protein-coding genes within the 8p23.3 duplicated region (*ZNF596*, *FBXO25*, and *TDRP*), we performed a gene-set analysis to provide additional evidence for a role of genetic variation in this region in ADHD and/or psychotic disorders. This was done using summary statistics of ADHD GWAS-MA data of 20,183 patients with ADHD and 35,191 controls (Demontis et al., 2017) and SCZ GWAS-MA summary statistics from the analysis of 34,241

cases and 45,604 controls (Schizophrenia Working Group of the Psychiatric Genomics, 2014). Joint analysis of the three genes showed significant association of the gene-set with ADHD in both self-contained and competitive tests ($P_{self\text{-}contained}$=0.000184 and $P_{competitive}$=0.039151; see also **Figure S2A**). No significance was observed for association with SCZ ($P_{self\text{-}contained}$=0.7123 and $P_{competitive}$=0.90596, see also **Figure S2B**).

To identify the most likely candidate gene for ADHD among the three protein-coding genes in the microduplication, the genes were subsequently tested individually. Gene-based analysis revealed that the two more proximal genes, *FBXO25* and *TDRP*, were significantly associated with ADHD (P_{FBXO25}=0.010756 and P_{TDRP}=0.000285; **Table S2**).

Brain gene expression

We determined the distribution of mRNA expression for *FBXO25* and *TDRP* in the developing and adult human brain using data on six brain regions available from the Human Brain Transcriptome Project. As the mRNA expression figures in **Figure S3** show, both genes appear to be widely expressed, both in the developing and the adult human brain.

Overexpression of the *FBXO25* orthologue in *Drosophila* induces increased activity and reduced sleep

To further establish the relevance of the associated genes as candidates for ADHD, we set out to study the effect of overexpression on ADHD-relevant behavioral phenotypes in *Drosophila melanogaster*. Only *FBXO25* has a direct orthologue (*CG11658*) in the fruit fly. We induced overexpression of *CG11658* using two unrelated *Drosophila* lines. Quantitative PCR confirmed the overexpression of this gene: in overexpression-line 1, expression of *CG11658* was 450% compared to the control line; in overexpression-line 2, it was 650%. Using our earlier established tests of locomotor activity and sleep as ADHD-relevant read-outs (van der Voet et al., 2016), we found adult flies from either line to exhibit significantly elevated activity and reduced sleep in the relative night period compared to the genetic background control (**Figure 3**). In line with the qPCR results, overexpression-line 2 showed the more severe behavioral phenotypes (**Figure 3**).

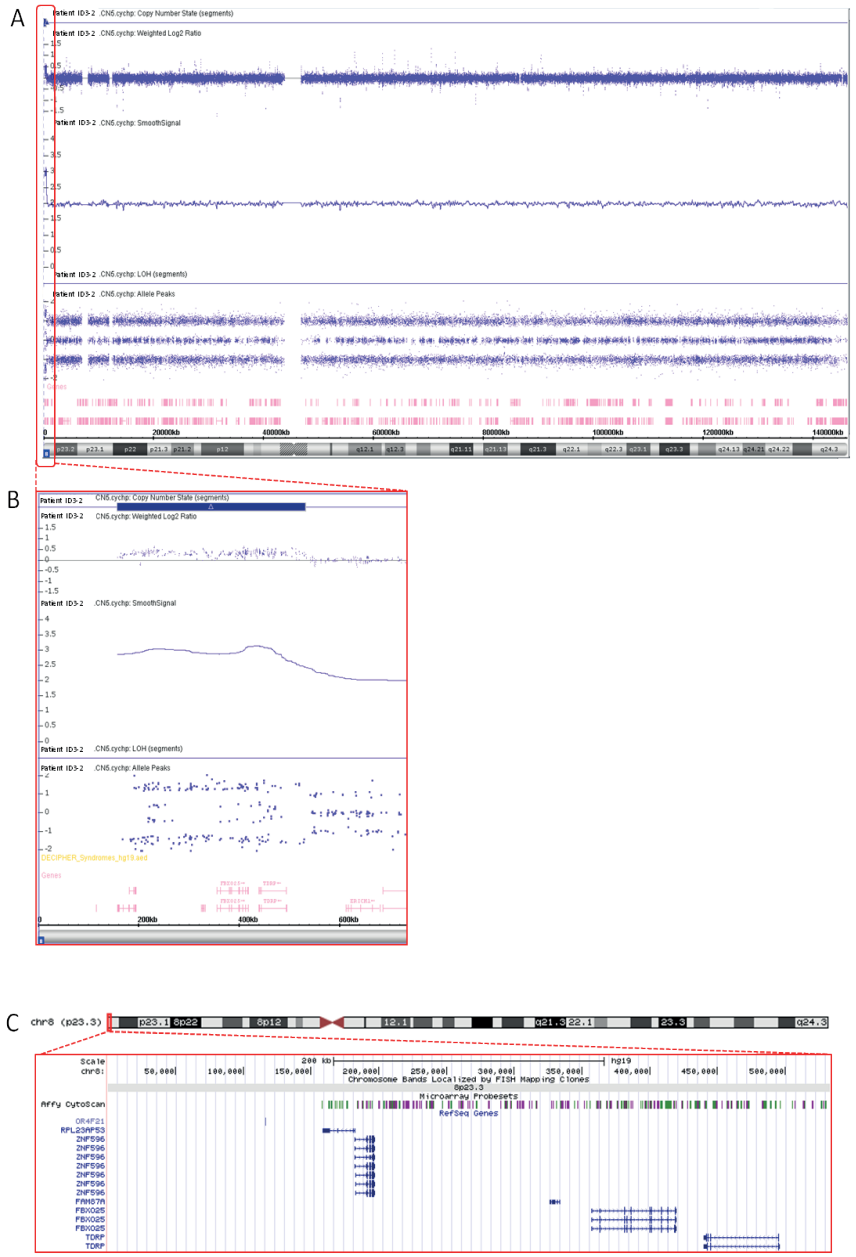

Figure 2: Microduplication of 8p23.3 in the index patient ID3-2. (**A**) Array CGH plot of chromosome 8 of patient ID3-2 with a terminal duplication of 535 kb in 8p23.3 spanning 590 probes (arr[hg19] 8p23.3(158,049–541,637)x3). Zoom-in of this region is shown in panel (**B**). (**C**) Schematic representation of chromosome 8 with the 8p23.3 region enlarged in the lower part of the figure (screenshot of the UCSC Genome Browser Build 37/hg19 (http://genome.ucsc.edu/)); the small vertical bars show the probe coverage of the CytoScan HD array platform, the horizontal bars represent the genes in the duplicated 8p23.3 region (*RPL23AP53*, *ZNF596*, *FBXO25*, and *TDRP*).

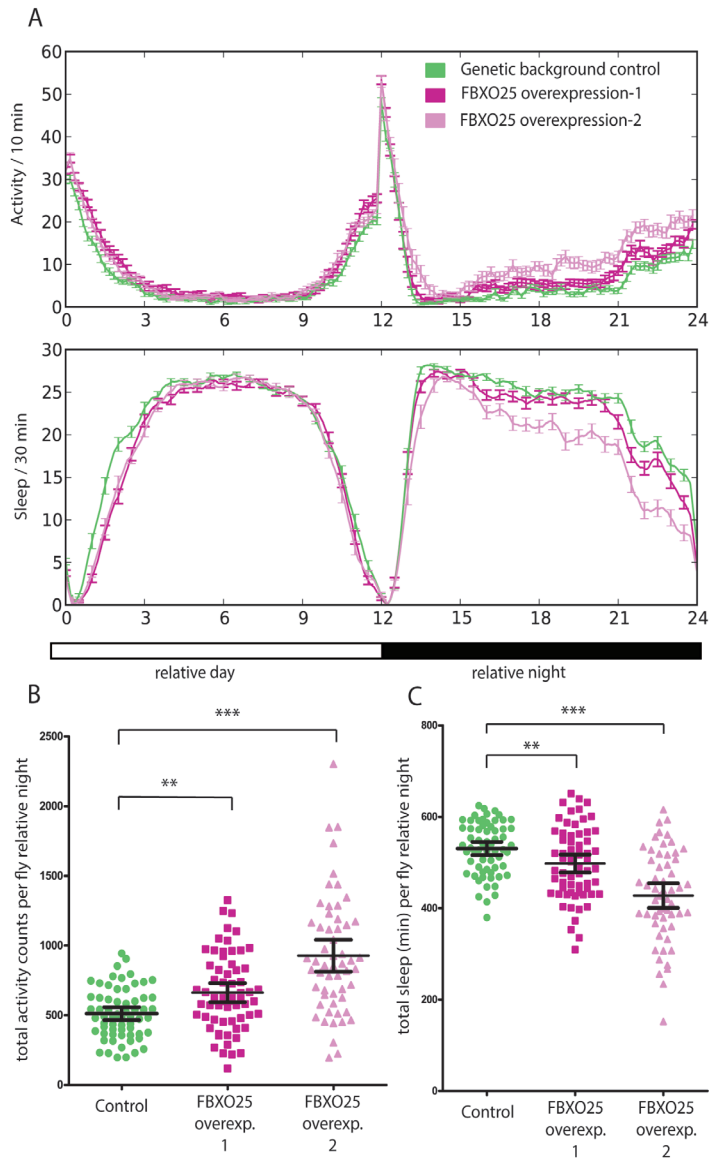

Figure 3: Activity monitoring upon pan-neuronal overexpression of the *FBXO25* orthologue in *Drosophila melanogaster*. (**A**) Flies show increased activity (top graph) and decreased sleep (bottom graph) in the relative night (12h–24h). (**B**) Quantification of the activity counts (amount of infrared detection beam passes) of individual flies in *FBXO25* overexpression–1 (n=64) and overexpression–2 (n=56) lines shows that the activity in the relative night is significantly higher in both compared to control (n=63) flies (*FBXO25* overexpression–1: P=0.0013; *FBXO25* overexpression-2: P<0.0001). (**C**) Quantification of sleep in minutes of single flies shows that both *FBXO25* overexpression lines sleep significantly less in the relative night compared to control flies (*FBXO25* overexpression–1: P=0.0076; *FBXO25* overexpression-2: P<0.0001). *P<0.05, **P<0.001, ***P<0.0001.

Whole-exome sequencing

Given the additional somatic features and early onset of severe psychiatric problems in the index patient, we performed clinical WES to identify additional rare variants that may contribute to the more severe clinical phenotype. Upon trio whole-exome data analysis of the index patient and his parents, one *de novo* candidate variant was found in the index patient. This was a heterozygous insertion variant in *DUSP16* (c.813_814insGC [p.Arg272fs] [GenBank: NM_030640.2]) leading to a frameshift; located at the end of exon 5 and it possibly affects the nearest splice site. This frameshift variant is not present in gnomAD (r.2.0.2). Exploratory gene-wide testing of common variants in *DUSP16* in the ADHD and SCZ GWAS-MA data did not reveal evidence for association with ADHD (P=0.39186), but the gene showed nominal association with schizophrenia (P=0.0175).

Discussion

In this study, we identified and functionally validated a novel candidate gene for ADHD and comorbid psychiatric phenotypes. We report a microduplication in band p23.3 of chromosome 8, which segregated with a broad spectrum of psychiatric problems, including ADHD, in a family with several affected individuals in two generations. Common genetic variants in this locus were associated with ADHD risk; in particular, we observed significant gene-based associations for the genes in the proximal part of the microduplication, *FBXO25* and *TDRP*. One out of the two genes has an orthologue in *Drosophila melanogaster*, *FBXO25*, and we were able to confirm that overexpression leads to a night-specific increase of locomotor activity and decrease of sleep. Since we have previously identified the same behavioral signature in several *Drosophila* models of ADHD (van der Voet et al., 2016), these findings suggest *FBXO25* as a novel risk gene for ADHD and comorbid mental disorders.
The identified microduplication in 8p23.3 has not previously been reported in conjunction with ADHD or other neuropsychiatric disorders. A recent study combined data from eleven CNV studies that had identified rare CNVs occurring in patients with ADHD (Harich et al., submitted). We queried these existing data sources and found an additional ADHD patient, among 6,176 reported ones, who carried a duplication encompassing 77% of the *FBXO25* gene. This duplication was not found in 25,026 controls (Elia et al., 2010; Harich et al., submitted).

The microduplication co-segregated with different psychiatric phenotypes, ranging from mild learning difficulties to ADHD, psychosis, substance use, and aggressive behavior. Of the two cousins of the index patient's mother, each with an ADHD diagnosis, one had a normal copy number for *FBXO25*, and for the second cousin no results were obtained due to poor quality DNA. Since ADHD is a rather common disorder in the population (Faraone et al., 2015), we can only speculate that there may be additional variants contributing to psychiatric

risk (e.g. coming from common variants (Demontis et al., 2017) and/or from environmental risk factors) present in this family, which contribute to the ADHD phenotype in addition to the structural variant. In that way, ADHD may not behave as a strictly monogenic disorder in any of the affected individuals in this pedigree, which is in line with findings from previous rare variant studies of the disorder (Corominas et al., in revision), and reminiscent of findings in previous linkage studies of ADHD (Lesch et al., 2008; Zhou et al., 2008) and other neurodevelopmental disorders (e.g. autism (Chapman et al., 2015)). Indeed, WES revealed an additional *de novo* frameshift variant in the *DUSP16* gene in the index patient, who also showed additional somatic features, anxiety disorder, and psychiatric episodes at very young age. A recent study reported that *Dusp16* plays a critical role in neurogenesis by balancing neural progenitor cell proliferation and neural differentiation (Zega et al., 2017). Moreover, mouse mutants lacking a functional *Dusp16* gene developed fully-penetrant congenital obstructive hydrocephalus together with brain overgrowth (Zega et al., 2017). When focusing on common genetic variants, we also found nominally significant association of *DUSP16* with schizophrenia. Therefore, we suggest that this *de novo* variant may have a modifying role in the early development of the patient's severe psychiatric phenotypes.

After association testing of the microduplication region in the data from the world-wide largest genome-wide association data set on ADHD risk, we identified two genes, the F-Box Protein 25 (*FBXO25*) and the Testis development related protein (*TDRP*) to be associated with ADHD risk. While both genes are expressed in the developing and adult brain, a function in the nervous system has not been described for either, thus far. *FBXO25* codes for an E3 ligase, which provides the substrate specificity for ubiquitin-dependent destruction or inhibition of transcription factors (Jang et al., 2011). *FBXO25* plays a role in cardiomyocyte development, and reduced expression of *FBXO25* appears to be involved in cell proliferation and migration in non-small-cell lung cancer (Jeong et al., 2015; Jiang et al., 2016). Knockdown of the gene in a cellular system has been linked to decreased phosphorylation of *ERK1*, whereas overexpression has an opposite effect on the MAPK signaling pathway (Teixeira et al., 2017). MAPK signaling plays a role in different psychiatric disorders, including ADHD and autism (Poelmans et al., 2013; Poelmans et al., 2011). Moreover, the brain relies on ubiquitination to fine-tune protein content in order to react to new stimuli (Hegde and van Leeuwen, 2017). Another E3 ligase, *FBXO33,* has been the top-finding of the first case-control genome-wide association study of persistent ADHD (Sanchez-Mora et al., 2015). Further F-box genes have been found associated with neurodevelopmental disorders, too, as e.g., reported in a patient with intellectual disability and seizures carrying a deletion of *FBXO28* (Au et al., 2014). *TDRP*, the second gene in the microduplication, is known to be involved in sperm motility (Mao et al., 2016). A rare missense variant in *TDRP* has been reported in monozygotic twins with gender dysphoria (Morimoto et al., 2017). Otherwise, little is known about this gene up to now.

Many different complex behaviors can be studied in *Drosophila*, and it is a model for a wide range of neuropsychiatric phenotypes (van Alphen and van Swinderen, 2013). Among the behaviors that can be robustly assessed in *Drosophila* are locomotor activity and sleep, which are known to undergo a well-regulated day/night rhythm. In a previous study, we reported a distinctive behavioral signature upon knockdown of ADHD candidate genes encoding the dopamine transporter, latrophilin, and neurofibromin 1, which included increased activity and decreased sleep, especially during darkness (van der Voet et al., 2016). Further experiments strongly suggested this phenotype to result from abnormal activity of the dopaminergic system. Upon overexpression of the *FBXO25* orthologue described here, we recapitulated this phenotype. This demonstrates that overexpression of *FBXO25* in the nervous system is sufficient to trigger increased activity, suggesting that it accounts or at least contributes to the ADHD-related phenotypes in our family. This is also the first report providing evidence for FBXO25 having a role in the nervous system.

The findings described here need to be interpreted in light of several strengths and limitations of our study. The main strength of this study is its interdisciplinary setting and comprehensive approach across multiple levels. For this, we integrated complementary evidence from genetic, genomic, and animal model experiments to examine the potential causes of complex psychiatric phenotypes including ADHD segregating across generations in a single family. We combined rare variants approaches (CNV analysis and WES) with an analysis of common variants in the identified CNV using the largest meta-analysis data sets currently available. For our functional validation of the identified candidate gene, we used an unconventional, well-validated animal model. A clear limitation of our study is that we were not able to test the effects of overexpression of *TDRP* on locomotion and sleep patterns in *Drosophila*, because this gene is not conserved in *Drosophila*. We were therefore not able to collect data that would argue for or against a contribution of *TDRP* overexpression to the complex psychiatric phenotypes seen in the family, in addition to *FBXO25*. Similarly, it would be of interest to investigate the role of *DUSP16* as a possible modifier of the phenotype of the index patient.

In conclusion, by integrating genetic and genomics studies with biological validation, we identified *FBXO25* as a novel risk gene for ADHD and comorbid psychiatric disorders. Future research is warranted to discover the underlying molecular mechanisms and identify proteins regulated by *FBXO25* in order to better understand the etiology of the aberrant behavior caused by variation in this gene.

Acknowledgements

The authors wish to thank all participants who took part in the study. This work was partly carried out on the Dutch national e-infrastructure with the support of SURF Foundation. BH was funded by a Radboud University Medical Center PhD grant. MvdV was supported by the Netherlands Organization for Scientific Research (NWO) Veni grant (91.614.084). BF and MK are supported by funding from a personal Vici NWO grant (016-130-669, to BF) and from funding for the Dutch National Science Agenda NeurolabNL project (grant 400-17-602). This work was also supported by a NWO TOP grant (912-12-109, to AS), the European Community's Seventh Framework Programme (FP7/2007 – 2013) under grant agreement n° 602805 (Aggressotype), and the European Community's Horizon 2020 Programme (H2020/2014 – 2020) under grant agreements n° 667302 (CoCA) and n° 643051 (MiND).

Conflict of interest

Barbara Franke received educational speaking fees from Shire and Medice. The other authors do not report biomedical financial interests or potential conflicts of interest.

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Supplementary methods

Study participants and cohorts

Clinical report of the Dutch family

We report a male index patient, born to non-consanguineous Dutch parents (Figure 1). Initially, he developed normally, however, at the age of two years, he became aggressive, hyperactive, and did not want to talk to others. The parents received video home training for his behavioral problems, which led to some improvement. When he went to school at the age of four years, he showed aberrant behavior. He had severe anxiety, could not be in large groups, showed aggressive behavior, and had to change school. He was diagnosed with ADHD and anxiety disorder by a pediatric psychiatrist. Later on, a diagnosis of multiple complex developmental disorder (MCDD) was made by another pediatric psychiatrist. Additionally, he suffered from a psychotic episode and showed suicidal tendencies. During his psychotic episode, he was hospitalized. The patient has an IQ of 95. At the age of 12 years, his behavioral problems became more severe, and he is currently living in a protected group home. The patient suffers from severe gastrointestinal reflux, for which he has undergone surgery at the age of nine years. He also suffers from severe constipation. The patient is treated with Concerta, Risperidon, and melatonin. His sleeping problems originated after the start of treatment with methylphenidate, but are now under control with melatonin. On physical examination, he had a length of 121 cm (-2.15 SD). His head circumference was 55.5 cm (+1.75 SD). He exhibits a triangular shaped face, an upturned nose, but otherwise has no remarkable facial dysmorphisms. He has clinodactyly of the 5th fingers. The family history showed that the mother has ADHD (formally diagnosed by a psychiatrist), whereas the father is not affected. The mother's brother suffered from a psychotic episode in the past and shows aggressive behavior. As a child, he was hyperactive and had concentration problems. He is addicted to alcohol and is homeless. He did not show dysmorphic features on physical examination by a clinical geneticist. The mother's sister did not have neuropsychiatric disorders, she did report mild learning difficulties, but completed regular education without extra support. The grandparents of the patient did not report any neuropsychiatric problems. However, two of the mother's cousins were diagnosed with ADHD at the Department of Psychiatry at the Radboud university medical center in Nijmegen, the Netherlands.

When the index patient was seen again by the clinical geneticist at the age of 12, his height was 142,8 cm (-2,3 SD), weight 35,5 kg (+0,7 SD) and his skull circumference 56 cm (+0,2 SD). He showed a triangular shaped face with minor dysmorphic features (broad front teeth, full lips, broad base to the nose). He had scapulae alatae and clinodactyly of the 5th fingers, but not other abnormalities.

Genome-wide ADHD GWAS-MA summary statistics data

ADHD GWAS-MA data were acquired from the ADHD Working Group of the PGC and the ADHD iPSYCH-SSI-Broad collaboration (n=55,374; (Demontis et al., 2017), https://www.med.unc.edu/pgc/results-and-downloads). Quality control and imputation were performed for each data set separately. Genotype imputation was with the pre-phasing/imputation stepwise approach implemented in IMPUTE2/SHAPEIT (chunk size of 3 Mb and default parameters) using the 1000 Genomes Project data set (phase 1, August 2012). After imputation, only autosomal SNPs with high imputation accuracy across all samples were taken forward. For association testing, all data sets were analyzed separately using an additive logistic regression model including ancestry principal components as covariates. Subsequently, meta-analysis of data sets was conducted using an inverse-weighted fixed effects model. In total, 20,183 cases and 35,191 controls were used for the original analysis. ADHD GWAS-MA data only included SNPs with an imputation quality score of INFO ≥0.8 and a minor allele frequency (MAF) ≥0.01 (8,047,420 SNPs).

Genome-wide schizophrenia GWAS-MA summary statistics data

SCZ GWAS-MA data were acquired from the SCZ Working Group of the PGC (n=82,315; (Schizophrenia Working Group of the Psychiatric Genomics, 2014), https://www.med.unc.edu/pgc/results-and-downloads). Quality control and imputation were performed for each data set separately. Genotype imputation was with the pre-phasing/imputation stepwise approach implemented in IMPUTE2/SHAPEIT (chunk size of 3 Mb and default parameters) using the 1000 Genomes Project data set (phase 1, August 2012). After imputation, only autosomal SNPs with high imputation accuracy across all samples were taken forward. For association testing, all data sets were analyzed separately using an additive logistic regression model including ancestry principal components as covariates. Subsequently, meta-analysis of data sets was conducted using an inverse-weighted fixed effects model. In total 34,241 cases and 45,604 controls were used for the original analysis. SCZ GWAS-MA data only included SNPs with an imputation quality score of INFO ≥0.8 and a minor allele frequency (MAF) ≥0.01 (9,444,230 SNPs).

Gene-set and gene-based analyses in the PGC/iPSYCH ADHD and PGC schizophrenia datasets

Statistical analyses were performed using the Multi-marker Analysis of GenoMic Annotation (MAGMA) software package (version 1.05; http://ctglab.nl/software/magma; (de Leeuw et al., 2015). Genome-wide SNP data from a reference panel (1000 Genomes, v3 phase1; (Genomes Project et al., 2010)) was annotated to NCBI Build 37.3 gene locations using a symmetric 100 kb flanking window. The gene annotation file was used to map GWAS-MA data (ADHD and SCZ separately), to assign genetic variants to genes, and to calculate gene-based p-values for each data set. For gene-based analyses, single variant p-values within a gene were

transformed into a gene-statistic by taking the mean of the χ2-statistic among variants in each gene. To account for LD, the 1000 Genomes Project European sample was used as a reference to estimate LD between variants within (the vicinity of) genes (http://ctglab.nl/software/MAGMA/ref_data/g1000_ceu.zip). Gene-wide p-values were converted to z-values reflecting the strength of the association of each gene with ADHD or SCZ risk, with higher z-values corresponding to stronger associations. Subsequently, we tested whether all protein-coding genes in the gene-set were jointly associated with ADHD or SCZ risk, using an intercept-only linear regression model including a subvector corresponding to genes in the gene-set. The *RPL23AP53* gene is a pseudo-gene and was therefore not included in the analyses. This self-contained analysis evaluated whether the regression coefficient of this regression was ≥0, testing whether the gene-set showed association with ADHD or SCZ risk. Next, we tested whether genes in this gene-set were more strongly associated with ADHD or SCZ than other genes in the genome. With this competitive test, differences between the association of the gene-set to all genes outside this gene-set is tested, accounting for the polygenic nature of a complex disorder like ADHD or SCZ. Potential confounders gene size and gene density, as well as their logarithms, were included in the competitive test. Because of this, we were more interested in the competitive than the self-contained test for the current analysis. Genes were considered gene-wide significant, if they reached the Bonferroni correction threshold adjusted for the number of genes within the gene-set (three tests; P<0.0167).

Copy number analysis in the Dutch family

The observed CNV was subsequently confirmed by real-time, quantitative PCR (qPCR) in the index patient and his mother, and three additional family members were analyzed using this method. Genomic DNA was either isolated from saliva or from EDTA blood samples according to manufacturer's protocol at the department of Human Genetics of the Radboud university medical center, Nijmegen, The Netherlands. PrimeTime® Mini qPCR assay (Integrated DNA Technologies [IDT], Coralville, IA, USA) was used to determine copy number of *FBXO25*. Primer sequences are displayed in Table S1. DNA concentrations were adjusted to 10 ng/µl before genotyping. RNAseP was taken along as endogenous control. Each qPCR reaction mix contained 0.25 µl *FBXO25/RNAseP* PCR primers, 5 µl TaqMan Universal PCR Master Mix (2x; Applied Biosystems Inc [ABI], CA, USA), 3.75 µl of water, and 1 µl genomic DNA (10 ng/µl). The amplification protocol for the reaction was 50°C for 2 min and 95°C for 10 min, followed by 95°C for 15 sec and 60°C for 1 min for 40 cycles. All measurements were performed in triplicate, and blanks were taken along as quality control during expression assessment. Results were analyzed with the 7500 Software v2.0.6 (Life Technologies) using an automatic threshold. As a calibrator sample the mean delta cycle threshold (ΔCT) of the control samples was used. Copy Caller software (ABI, version 2.0) was used to calculate the integer copy number of each probe based on the real-time PCR data. The mean and standard

deviation (SD) of triplicates of ΔCT for each subject was calculated. Data was visualized using GraphPad prism (version 5.03), and the mean and a 95% confidence interval are shown.

Supplementary tables

Table S1. Primer sequences for quantitative PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
FBXO25	GTCTTCACTAAGCATATACAACGTG	ACCTTCCTCTGCACATGC
RNAseP	AGGGACATGGGAGTGGAG	CTGGGAAATCACCATAAACGTG
d-eIF-1A_rg	ATCAGCTCCGAGGATGACGC	GCCGAGACAGACGTTCCAGA
d_ αTub84B -alpha-tubulin_rg	TGTCGCGTGTGAAACACTTC	AGCAGGCGTTTCCAATCTG
dFBXO25_CG11658	GTGAGGCTGTGAAGCGTCT	GGCTGTCATTTACATGCGAGG

Table S2. Gene-based association results for the three protein-coding genes within the 8p23.3 locus using data from the ADHD GWAS-MA (Demontis et al., 2017).

Gene	Start	Stop	N SNPs	Zstat	P
ZNF596	82200	297339	646	1.8938	0.029127
FBX025	256808	519876	1295	2.2989	0.010756*
TDRP (C8orf42)	339790	595781	1195	3.4458	0.000285*

*Significant association after Bonferroni correction (three tests, P<0.0167).

Supplementary figures

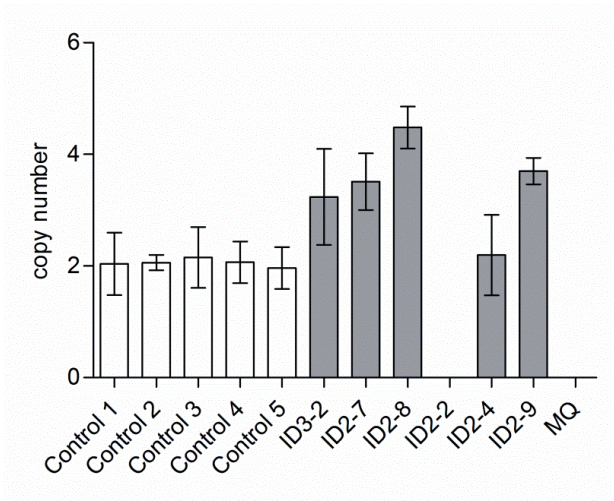

Figure S1: Validating gain of copy number of *FBXO25* gene by PrimeTime® Mini qPCR assay. Patient IDs correspond to IDs presented in Figure 1. Bar charts represent mean and 95% confidence interval. Copy number has been calculated from raw results by using the CopyCaller software (ABI, version 2.0). The patient's father DNA sample was not available for testing. DNA of ID2-2 was of too poor quality to be analyzed.

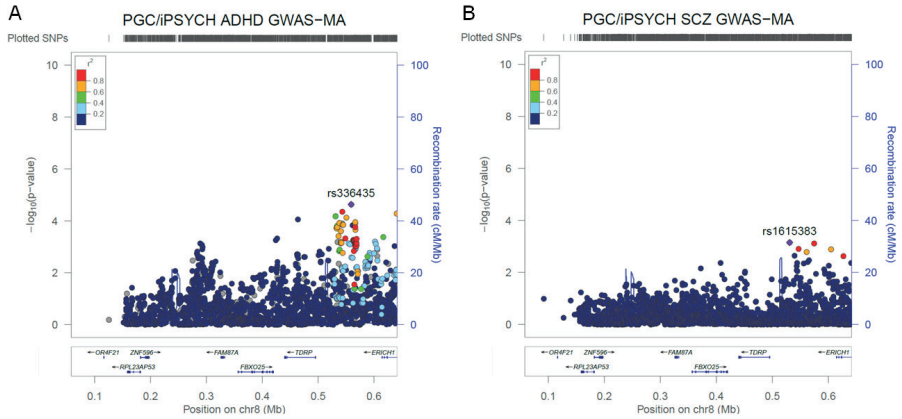

Figure S2: Regional association plot for 8p23.3 locus (±100 kb) using (**A**) the ADHD GWAS-MA data (Demontis et al., 2017) and (**B**) the schizophrenia GWAS-MA data (Schizophrenia Working Group of the Psychiatric Genomics, 2014). Results are shown as –log (p value) for genotyped and imputed single nucleotide polymorphisms (SNPs). The color of the markers reflects local linkage disequilibrium (r2) of the strongest associated SNP (indicated by the purple dot), as shown in the legend. The local recombination rate is depicted by the bright blue peaks (magnitude indicated by the right-hand y axis). cM/Mb, centimorgan/megabase. Chr, chromosome. Plot was made using the Locuszoom software (Pruim et al., 2010).

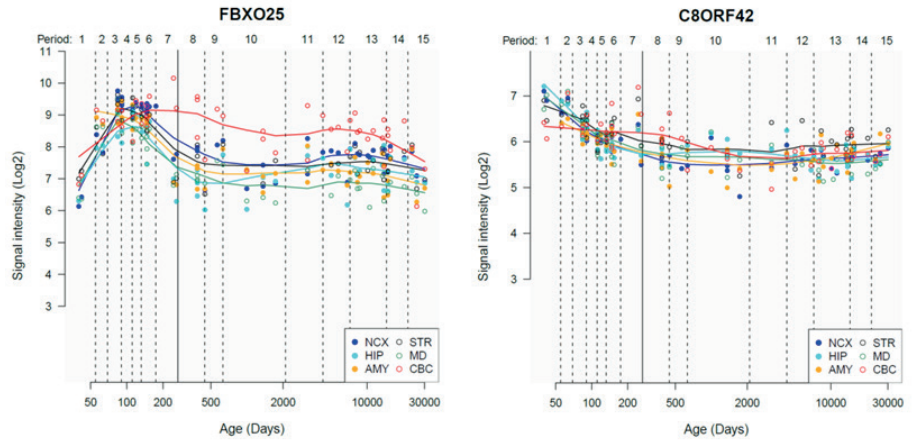

Figure S3: Expression trajectories of *FBXO25* and *TDRP (C8orf42)* in the developing and adult human brain. Line plots show the $\log_2$-transformed gene exon array signal intensity from the early fetal period to late adulthood in six brain regions. The solid line between periods 7 and 8 (approximately post-conception day 280) separates prenatal from postnatal periods. Data were generated using Affymetrix GeneChip Human Exon 1.0 ST Arrays by the Human Brain Transcriptome project, and accessed via their publicly available database at http://hbatlas.org (Kang et al., 2011). Abbreviations: NCX=neocortex; HIP=hippocampus; AMY=amygdala; STR=striatum; MD=mediodorsal nucleus of the thalamus; CBC=cerebellar cortex.

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CHAPTER 6

Identification of ADHD candidate genes in large pedigrees by combining linkage analysis and whole exome sequencing

Published as:

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Abstract

Attention-Deficit/Hyperactivity Disorder (ADHD) is a common neurodevelopmental disorder with a complex genetic background, hampering identification of underlying genetic risk factors. We hypothesized that combining linkage analysis and whole exome sequencing (WES) in multi-generation pedigrees with multiple affected individuals can point towards novel ADHD genes. Three families with multiple ADHD-affected members (N_{total}=70) and apparent dominant inheritance pattern were included in this study. Genotyping was performed in 37 family members, and WES was additionally carried out in 10 of those. Linkage analysis was performed using multi-point analysis in Superlink Online SNP 1.1. From prioritized linkage regions with a LOD score ≥2, a total of 24 genes harboring rare variants were selected. Those genes were taken forward and were jointly analyzed in gene-set analyses of exome-chip data using the MAGMA software in an independent sample of patients with persistent ADHD and healthy controls (N=9,365). The gene-set including all 24 genes together, and particularly the gene-set from one of the three families (12 genes), were significantly associated with persistent ADHD in this sample. Among the latter, gene-wide analysis for the *AAED1* gene reached significance. A rare variant (rs151326868) within *AAED1* segregated with ADHD in one of the families. The analytic strategy followed here is an effective approach for identifying novel ADHD risk genes. Additionally, this study suggests that both rare and more frequent variants in multiple genes act together in contributing to ADHD risk, even in individual multi-case families.

Keywords: ADHD, genetics, linkage analysis, whole-exome sequencing, *AAED1*

Introduction

Attention-Deficit/Hyperactivity Disorder (ADHD) is a multifactorial neurodevelopmental disorder, characterized by age-inappropriate inattention, hyperactivity and increased impulsivity. ADHD is frequent in children, and in up to 60% of the cases impairments persist into adulthood (Faraone et al., 2006). ADHD presents a high risk for developing co-morbid disorders, increasing the burden on social, educational, and professional aspects of life (Biederman, 2004; Faraone et al., 2015). Family and twin studies showed that ADHD is highly heritable, both in childhood and in adulthood, with heritability estimates range between 70 and 90% (Chang et al., 2013; Faraone et al., 2005; Larsson et al., 2014). Despite this considerable heritability, the identification of risk genes has been challenging (Faraone et al., 2015; Franke et al., 2012), and one reason for this could be the genetic complexity of the disease. Identified candidate genes so far mainly belong to monoaminergic neurotransmitter pathways, especially dopaminergic and serotonergic signaling (Bralten et al., 2013; Franke et al., 2010; Gizer et al., 2009; Weber et al., 2015; Wu et al., 2012). Different (hypothesis-free) approaches, including genome-wide linkage analyses and genome-wide association studies (GWASs), have been performed in order to detect additional genetic factors for ADHD. In line with the 'common disease-common variant' model, mostly common genetic factors have been investigated, which generally convey very small effect sizes (Faraone et al., 2015). However, GWASs of ADHD are only just reaching sufficiently large samples sizes to produce genome-wide significant results. Linkage analysis, a method useful for identification of genetic risk factors of larger effect size using family data, has also contributed to the identification of risk loci for ADHD. A meta-analysis of linkage studies in ADHD reported a significant region in the distal part of chromosome 16q (Zhou et al., 2008). Within this region, the *CDH13* gene was repeatedly found among the top-findings in GWASs (Lesch et al., 2008). In addition, linkage analysis in families from a genetic isolate in combination with association testing identified the *ADGRL3/LPHN3* gene as an ADHD risk factor (Arcos-Burgos et al., 2010; Ribases et al., 2011). More evidence for involvement of less frequent genetic variants with potentially larger effect sizes comes e.g. from genome-wide studies of copy number variants (CNVs) (Lesch et al., 2011; Merker et al., 2017; Thapar et al., 2016; Williams et al., 2012; Williams et al., 2010) and initial exome-chip (Zayats et al., 2016) and whole-exome sequencing work (Demontis et al., 2016; Kiser et al., 2015). In addition, whole-exome sequencing (WES) has been successful in identifying rare risk alleles for other neurodevelopmental/psychiatric disorders, such as autism spectrum disorders (ASDs) and schizophrenia (e.g. (Iossifov et al., 2014; Purcell et al., 2014)).

In this study, we explored whether a combination of linkage analysis and WES in large multi-generational pedigrees is a viable approach to gene-finding in ADHD. We narrowed down the search area for rare variants by linkage analysis in three multi-generation pedigrees with multiple ADHD-affected members. Based on the WES applied to subsets of

family members, we selected rare variants present in all (suggestive) linkage regions in each family. In line with the polygenic nature of ADHD, in which both common and rare genetic variants are likely to contribute to disease etiology, we subsequently used the extracted gene-sets to analyze the cumulative role of common and rare variants in persistent ADHD in an independent exome-chip data set (IMpACT consortium; N=9 365 (Zayats et al., 2016)).

Materials and methods

Study participants

Multigenerational pedigrees

The study included three multi-generational families with multiple ADHD affected individuals (N_{total}=70, N_{ADHD}=41). The structure of the three families (Pedigree 1-3; P1-P3) is summarized in **Table 1** and shown in **Supplementary Figure 1**. All families were of German origin and were ascertained through affected children referred to the outpatient clinic of the Department of Child and Adolescent Psychiatry and Psychotherapy, University Hospital Würzburg, Germany. For the index-child, strict inclusion and exclusion criteria were applied. Included index-children were aged ≥6 years and met criteria for ADHD combined subtype according to DSM-IV. Index-children had a birth weight >2,000 grams and Intelligence Quotient (IQ) >80, did not show any neurological or severe somatic disorder, drug abuse or ASDs, and did not receive psychotropic medication (except for stimulant medication for ADHD). Detailed description of the diagnostic procedure for family members was reported previously (Romanos et al., 2008). The study was approved by the Ethics Committee of the Julius-Maximilians-University of Würzburg. Written informed consent was obtained from all participating individuals.

Table 1. Summary of the families included in this study.

Family	Total	Affected	Unaffected	Unknown	WES	Genotyping
P1	11	9	1	1	5	7
P2	29	15	6	8	2	15
P3	30	17	8	5	3	15

WES=whole-exome sequencing; genotyping indicates the number of family members with available genome-wide genotyping data for linkage analyses.

Exome-chip data set

The data set, which did not include members of the families above, was genotyped on the Infinium Human CoreExome chip (Illumina, San Diego, CA, USA) and comprised 1,846 adults with persistent ADHD and 7,519 controls recruited from four different countries: Spain (615 cases and 932 controls), Norway (597 cases and 2,598 controls), Germany (340 cases and 2,286 controls), and The Netherlands (294 cases and 1,703 controls). Part of the Dutch controls were derived from the Nijmegen Biomedical Study (NBS, www.nijmegenbiomedischestudie.nl), a population-based survey conducted by the Departments of Epidemiology & Biostatistics and Clinical Chemistry of the Radboud University Medical Center (Galesloot et al., 2017). Part of the Norwegian controls were derived from The Nord-Trøndelag Health Study (The HUNT study), a large population-based cohort (Krokstad et al., 2013). Part of the German controls were derived from the Heinz-Nixdorf-Recall cohort, a large population based cohort (Schmermund et al., 2002). Persistent adult ADHD was diagnosed according to DSM-IV criteria. A detailed description of all samples and (genotyping) procedures was recently published (Zayats et al., 2016), and a shortened version is included in the **Supplementary Methods**. ADHD cases were of European descent and were part of the International Multicenter persistent ADHD Collaboration (IMpACT (Franke and Reif, 2013)). The study was approved by the Ethics Committees of the respective universities and/or hospitals. All participants signed informed consent.

Single Nucleotide Polymorphism (SNP) Genotyping and Linkage Analysis

Genome-wide SNP genotyping was performed on Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, USA). Microarray quality control parameters and genotype calls were generated with Affymetrix Genotyping Console v4.2.0.26 software (call rate >0.99). Individuals were excluded if their call rate was below 97%. Genotyping data were filtered by removing SNPs with minor allele frequency (MAF) <5%, missing genotypes >5%, Mendelian errors >10% for variants, or deviations from Hardy-Weinberg equilibrium (HWE, $P \leq 10^{-6}$). The remaining 665,362 SNPs were pruned to reduce linkage disequilibrium (LD) between markers using PLINK v1.07 software (http://pngu.mgh.harvard.edu/purcell/plink/ (Purcell et al., 2007)) with pairwise R^2 >0.01 in sliding windows of 50 SNPs, moving in intervals of five SNPs. In total, 10,842 autosomal SNPs were included in the linkage analyses. Multi-point linkage analyses of genome-wide SNP data were performed using Superlink Online SNP 1.1 (Silberstein et al., 2013), which can handle large pedigrees. Through parametric analysis, we computed logarithm of odds (LOD) scores using groups of markers with a window size of 10 markers. Based on the phenotypic characteristics of the pedigrees, a dominant model was specified with an allele frequency of 0.01 and a penetrance value of 0.99. The significance level was set at a LOD score of 3.3, but a score ≥2 was used to select candidate regions for further analyses (**Supplementary Figure 2**). The linked regions were bounded using 1 LOD-score intervals upstream and downstream from the marker with the highest LOD-score. Haplotypes of the regions of interest were constructed by using the haplotyping tool of Superlink Online SNP 1.1 (Silberstein et al., 2013).

Whole-Exome Sequencing (WES)

For each family, two or more affected family members were strategically selected for WES based on meiotic distance and/or position in the pedigree (**Table 1** and **Supplementary Figure 1**). Genomic DNA was extracted from blood samples according to standard protocols (Romanos et al., 2008). The exome was targeted by Agilent Sure Select Human All Exon 50 Mb Target Enrichment kit (Agilent Technologies, Santa Clara, CA, USA) and sequenced by single-end sequencing on the 5500xl SOLiD™ System (Life Technologies, Carlsbad, CA, USA). High quality reads were mapped to the hg19 reference genome (UCSC genome browser) using the Lifescope 2.1 software (http://www.lifetechnologies.com/lifescope/) with default parameters. In addition, the SOLiD Lifescope Software v2.1 was used to call single nucleotide variants (SNVs) using the diBayes algorithm. Variant annotation was done at the Department of Human Genetics of the Radboud university medical center using a pipeline developed in-house (de Ligt et al., 2012). Variants were selected according to the following criteria: i) variants with ≥20 reads, ii) variants with a MAF <1% in dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), and iii) variants present in all sequenced family members. Functional effects of variants were predicted by three different softwares: SIFT (Sim et al., 2012), PolyPhen-2 (Adzhubei et al., 2010), and MutationTaster (Schwarz et al., 2014). Conservation of variants was estimated by PhyloP (Pollard et al., 2010) and Grantham score (Grantham, 1974).

Gene-based and gene-set analyses in the exome-chip data set

The cumulative effect of common and rare variants in selected genes and gene-sets was tested using the independent, recently published IMpACT exome-chip data set (**Supplementary Methods**; (Zayats et al., 2016)). Statistical analyses were performed using the Multi-marker Analysis of GenoMic Annotation (MAGMA) software package (version 1.02; http://ctglab.nl/software/magma (de Leeuw et al., 2015)) and the SNP-wide mean model for gene-based association analysis (for detailed description see **Supplementary Methods**). Importantly, all data sets (Spanish, Norwegian, German, and Dutch) were analyzed individually, followed by meta-analysis on the level of gene-based statistics. Fixed effects were used to perform meta-analysis by using the square root of the sample sizes as weights. An LD correction was applied, based on the estimates of the 1000 genome phase 1 European ancestry samples (Genomes Project et al., 2010). Data was analyzed following a step-wise approach (see also **Supplementary Figure 2**): First, an overall gene-set was created, including all genes identified by the approach described above (24 genes). We tested whether all genes together were jointly as a gene-set associated with persistent ADHD. Post-hoc to the analysis of the overall gene-set, we also tested family-specific gene-sets to localize the effect. Subsequently, we performed a gene-based look-up of genes from family P2 (12 genes), and genes were considered gene-wide significant, if they reached the Bonferroni-corrected threshold adjusted for the number of genes within the gene-set (12 tests; P<0.0042).

Gene Ontology (GO) enrichment analysis

To assess whether the 12 prioritized genes from family P2 converge on biological shared functions, we tested for enrichment in Gene Ontology (GO) terms for biological processes using FUMA (Watanabe et al., 2017). Overrepresentation of biological functions of prioritized genes was tested for by comparison with gene-sets obtained from the Molecular Signature Database (MsigDB) v5.2 (i.e. GO gene sets), using hypergeometric tests. The sets of background genes were derived from 19,264 protein-coding genes. Benjamini-Hochberg correction (FDR) was used for multiple test correction method for gene-set enrichment testing. Gene-set enrichments were considered significant at an adjusted P-value level <0.05.

Segregation analysis in family P2

To validate the presence of selected rare variants in the two sequenced individuals and to allow segregation analysis, all individuals of family P2 were genotyped for chr9:99404124G>C (rs151326868) and chr8:124346225T>C using PCR-based DNA sequencing. The locus of interest was amplified by conventional PCR and sequenced by direct Sanger sequencing (details and primer sequences are available upon request). Data obtained for the two variants was used to analyze the segregation with ADHD diagnosis.

Results

Linkage Analysis across three families with ADHD

The main aim of the linkage analyses was to provide an additional filtering step for the WES data by narrowing down the genomic regions of interest. Linkage analysis was performed for each family individually, but also for all possible combinations of the three families. Informative individuals from each family were enrolled in the linkage study: nine individuals from family P1, 19 individuals from family P2, and 16 individuals from family P3 (**Supplementary Figure 1**). A total of 13 linkage regions with LOD-score ≥2 on chromosomes 6, 8, 9, 10, 11, 13, and 16 were identified (**Supplementary Table 1, Supplementary Figure 3 and 4**), and all were taken forward for subsequent analyses. Several significantly linked regions were observed by analyzing families together. The highest LOD-scores (3.99 and 3.79) were located on chromosome 16 in the analysis combining P1 and P3 (**Supplementary Table 1, Supplementary Figure 3 and 4**). In family P1, six linkage regions were selected (LOD-score ≥2) for further analyses, but all of them needed the contribution of at least one additional family to reach significance. In family P2, nine linkage regions were identified, three of which specific to this family, and in family P3, nine linkage regions were identified of which five were specific to this single family (all linkage regions with LOD-score ≥2 ; **Table 2** and **Supplementary Table 1**).

Table 2. List of candidate regions and genes selected based on the linkage analysis in each family. Genes were included if they were present in the linkage region (LR; ± 1Mb) with LOD ≥2, to which), the family was contributing and if they harbored a rare variant (according to our selection criteria).

Family	LR selected	Genes with rare variants in WES	Gene-set analysis	
		$P_{self\text{-}contained}$	$P_{competitive}$	
P1	8:118608158-124649389	-----	0.2838	0.4512
	9:7754113-15568230	*TYRP1*, *FREM1*		
	9:97466973-102213749	-----		
	11:115218677-120365028	*NXPE1*,*TMEM25*, *HYOU1*, *VPS11*, *ABCG4, CCDC153*		
	16:63079319-66386711	*HSF4*		
	16:81159781-83154022	*DYNLRB2*, *PKD1L2*, *PLCG2*, *OSGIN1*, *MBTPS1*		
P2	8:118608158-124649389	*DEPTOR*, *ATAD2*	0.0066	0.0042
	9:7754113-15568230	*PTPRD*, *TYRP1*, *FREM1*		
	9:97466973-102213749	*HSD17B3*, *AAED1*, *ANP32B**, *TBC1D2*		
	10:56177098-58789387	*PCDH15*		
	10:64668048-65875491	-----		
	11:21968768-29134515	*ANO3*		
	11:115218677-120365028	*BUD13*, *VPS11*		
	13:106701406-109091885	-----		
	16:63079319-66386711	-----		
P3	6:203878-460901	-----	0.1368	0.1393
	6:3446942-4470581	-----		
	9:97466973-102213749	-----		
	10:14311273-15844850	-----		
	10:64668048-65875491	-----		
	11:115218677-120365028	*BUD13*, *TMEM25*, *VPS11*		
	13:106701406-109091885	-----		
	16:63079319-66386711	*CDH5*		
	16:81159781-83154022	*MBTPS1*		

*No variants were observed in the *ANP32B* gene in IMpACT exome-chip data. Gene-set-based association analysis used meta-analytic exome-chip data from 9,365 individuals (1,846 ADHD patients and 7,519 controls (Zayats et al., 2016)).

Whole-exome sequencing analysis

A total of ten ADHD-affected family members were included in WES: five from family P1 (ID1, ID2, ID4, ID5, and ID11), two from family P2 (ID21 and ID26), and three from family P3 (ID17, ID19, and ID20)) (**Supplementary Figure 1**). We obtained an average of 5.46 billion bases of sequence per individual and about 82.2% (approximately 4.49 billion bp) of the total bases mapped to the exomes, with a mean of 85-times coverage (for WES sequencing statistics per individual see **Supplementary Table 2**). Based on our selection criteria, the average number

of shared rare variants present in each family was 1 235 across the exome. Applying filtering based on the identified linkage regions, a total of 20 variants were selected from family P1, 13 variants from family P2, and five variants from family P3 (**Supplementary Table 3**).

Association analyses and candidate gene identification in an independent sample

All genes within linkage regions containing at least one of the selected rare variants were included in a list of candidate genes (**Table 2**). Gene-set analysis was performed based on this list using exome-chip data from an independent sample of 1,846 adults with persistent ADHD and 7,519 controls (Zayats et al., 2016). Following testing of the overall gene-set (24 genes), we also tested gene-sets resulted from each family separately (**Table 2**). Meta-analysis of the individual exome-chip samples showed significant association of the overall gene-set in both self-contained and competitive tests ($P_{self\text{-}contained}$=0.0063 and $P_{competitive}$=0.0103, **Table 2**). The significant effect of the general gene-set was mainly driven by the effect of genes selected based on the linkage analyses in family P2 ($P_{self\text{-}contained}$=0.0066 and $P_{competitive}$=0.0042, 12 genes, **Table 2**), with additional minor contributions of the gene-sets resulting from the linkage analysis in the other two families (**Supplementary Table 4**). Focusing on individual genes of the P2 gene-set, gene-based analysis revealed that the *AAED1* gene was significantly associated with persistent ADHD (P=0.0039). Another gene in this gene set - *ATAD2* - yielded suggestive significance after correction for multiple testing (P=0.0072, **Table 3**). For both genes, association was driven entirely by rare variants (**Supplementary Table 5** and **6**).

To assess whether the 12 prioritized genes of the family P2 gene-set converged on biological functions or pathways, we tested for enrichment in GO-terms (biological processes). Four significantly enriched GO-terms were detected, including 'regulation of vesicle fusion' ($P_{adjusted}$=0.0166) and 'cell-cell adhesion via plasma membrane adhesion molecules' ($P_{adjusted}$=0.0328) (**Supplementary Figure 5**).

Table 3. Gene-based association results for the family P2 gene-set using IMpACT exome-chip data of 9,365 individuals (1,846 ADHD patients and 7,519 controls; (Zayats et al., 2016)).

Gene	N variants	P
AAED1	5	0.0039*
ATAD2	5	0.0072
BUD13	11	0.0136
ANO3	9	0.1308
DEPTOR	6	0.2279
TYRP1	8	0.2824
TBC1D2	12	0.3181
VPS11	9	0.3258
PCDH15	27	0.3434
PTPRD	28	0.6350
HSD17B3	5	0.7097
FREM1	23	0.9460

*Significant association after Bonferroni correction (12 tests, P<0.00417).

Single variant validation and familial segregation analysis

Going back to the WES data of family P2, one rare missense variant was identified in both candidate genes (*AAED1* and *ATAD2*) from the gene-based analysis. The variant rs151326868, located in *AAED1* (chr9:99404124G>C), was predicted to be deleterious in all pathogenicity tests (Polyphen2, SIFT, and MutationTaster), was highly conserved (PhyloP >2.7 and Grantham score >80; **Supplementary Table 7**), and rare (MAF=4.38x10^{-4} in the ExAC browser). The SNV chr8:124346225T>C in *ATAD2* was predicted to be deleterious only by MutationTaster, showed low conservation scores, and had very low MAF in the ExAC browser (8.24x10^{-06}; **Supplementary Table 7**). The variant rs151326868 in *AAED1* was also present in the exome-chip data (exome-chip marker exm764638; **Supplementary Table 5** and **6**), the SNV chr8:124346225T>C in *ATAD2* was not.

Sanger sequencing of these two rare variants in *AAED1* and *ATAD2* in all members of family P2 for whom DNA was available confirmed the presence of these variants in the two sequenced individuals and allowed segregation analysis. None of the healthy individuals carried either of the variants, 93% of the affected individuals (14/15) carried at least one of the two variants, and 60% of the affected individuals (9/15) carried both variants (**Figure 1**).

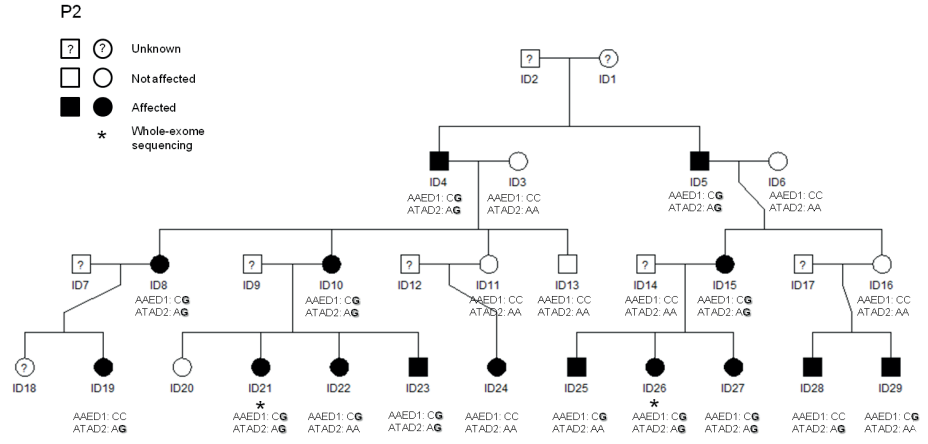

Figure 1. Segregation analysis for rs151326868 (chr9:99404124G>C; *AAED1* gene) and the SNV at chr8:124346225T>C (*ATAD2* gene) in family P2. ADHD-affected individuals are depicted by black symbols, unaffected family members are shown by white symbols and individuals with unknown ADHD status are represented by a question mark in the symbol. An asterisk beneath an individual indicates that DNA was used for whole-exome sequencing analysis. Non-reference alleles are depicted in bold.

Discussion

In this study, we aimed to identify novel genetic risk factors for ADHD by combining linkage analyses and WES in multi-generation families. We predicted that through a family-based approach, we would be able to limit genetic heterogeneity, since rare variants with potentially higher penetrance may cause the disorder in familial ADHD cases. Linkage analyses revealed four genomic regions with a LOD score ≥3.3 and 15 additional regions with a LOD score ≥2. Within these regions, we identified 38 rare variants within 25 genes across the three families. All genes together, and particularly the gene-set from family P2, were significantly associated with persistent ADHD in the independent exome-chip sample. Moreover, the *AAED1* gene reached gene-wide significance in that sample, and a rare variant in *AAED1* (rs151326868) segregated with ADHD in family P2.

Different designs can be used for WES studies aiming at identifying rare variants linked to complex diseases. In schizophrenia (Purcell et al., 2014) and ASDs (Neale et al., 2012; O'Roak et al., 2012; Sanders et al., 2012), large sample sizes of cases and controls have been used to find genes implicated in disease through rare variants in multiple patients. For ADHD, data sets have not yet reached the critical size to allow for a genome-wide, hypothesis-free analysis of WES data, but an initial study analyzed a pre-defined gene-set of interest and found evidence for enrichment of rare variants in cases (Demontis et al., 2016). A second approach, that has successfully been applied in other neurodevelopmental disorders, especially in ASDs (Hashimoto et al., 2016; O'Roak et al., 2012), is a trio-approach in sporadic

patients and their parents. In this approach, one is assuming that the occurrence of the disorder in the patient is due to a *de novo* mutation. In ADHD, this design may seem less promising since the disorder does not reduce reproductive fitness as it does in ASDs (Ploeger and Galis, 2011) and, therefore, sporadic cases are less frequently described and familial aggregation of ADHD is frequently observed (Chen et al., 2016). However, recent evidence from Swedish population registries suggests that ADHD risk is strongly increased in the offspring of fathers older than 45 years (D'Onofrio et al., 2014), which seems to be linked to an age-related increasing mutation rate in the paternal germline (Kong et al., 2012). Our own work also suggests that the cognitive profile of families with only one affected individual differs from that of families with more cases (Oerlemans et al., 2015a; Oerlemans et al., 2015b), which may suggest that the trio-design could also be successful in ADHD. The third design, which we employed in the current study, is the extended pedigree-based approach, in which one screens for segregation of rare variants with disease across multiple affected individuals. Knowledge on the etiology of ADHD is, however, still limited, and therefore, ranking and prioritization of potential candidate genes is challenging. With this in mind, our combined linkage and WES approach did help to efficiently limit the list of potentially causative variants in a data-driven way. Filtering WES variants by linkage analysis has earlier been shown to be an effective tool for prioritizing common and exome variants in extended families with ADHD (Lesch et al., 2008) or ASD (Chapman et al., 2015).

We extended the family-based approach by testing the effects of observed genes carrying rare variants in an independent, large sample of exome-chip data. Importantly, most of the selected rare variants in the genes included in the gene-set analysis of family P2 are exonic and non-synonymous variants, so the overall result in the case-control analyses would not be affected by more stringent selection criteria for rare variants that are frequently used in WES studies (e.g., being functionally relevant). Specifically, the significant gene-based association of *AAED1* would remain. Utilizing this independent sample, we showed that (some of the) identified genes may be relevant to ADHD in the population, thereby generalizing the findings from the single family. This approach also enabled us to study the cumulative effect of rare and common genetic variants in the identified candidate genes for association with persistent ADHD, maximizing power to find association by taking into account allelic heterogeneity (Bralten et al., 2013; Zayats et al., 2016).

Importantly, our work supports the notion that - despite the apparent dominant segregation pattern - ADHD is not a monogenic disorder in the pedigrees investigated. Linkage analyses revealed several (suggestive) signals per family, suggesting that several genes/loci may carry risk variants for ADHD in each of those. Based on the linkage analyses, we did not expect a single gene or single locus to be associated with the clinical phenotype nor perfect co-segregation pattern of the rare, non-reference allele with ADHD in subsequent segregation analyses. A main contributing factor to the observed patterns may be assortative mating, which is common in ADHD (Boomsma et al., 2010) (and e.g. present in

family P1). Although we were quite liberal in selecting regions for further analysis (through including suggestive linkage signals), the observed pattern is similar to findings in previous linkage studies of ADHD (Lesch et al., 2008; Zhou et al., 2008) and other neurodevelopmental disorders (e.g. for ASDs (Chapman et al., 2015)).

The prioritized genes in the gene-set of family P2 converged on the biological function of vesicle fusion, which adds to the relevance of our findings, since the process of vesicle fusion to plasma (e.g., synaptic) membrane is closely related to the mechanism of neurotransmitter release. The *AAED1* gene (coding for the AhpC/TSA Antioxidant Enzyme Domain Containing 1 protein) was significantly associated with persistent ADHD in the exome-chip sample, and the rare variant in this gene (rs151326868; MAF for C-allele in ExAC=$4.38x10^{-4}$) segregated with ADHD risk in family P2. AAED1 strongly binds and interacts with the Protein Kinase C-Alpha-Binding Protein (PICK1) (Huttlin et al., 2015). PICK1 binds to the dopamine transporter (DAT), more specifically to its carboxyl terminus, and is an important regulator of DAT trafficking in presynaptic sites of dopaminergic neurons (Torres, 2006). Additionally, a direct and functional interaction between PICK1 and dopamine D_3 receptors (D_3R) has been reported (Zheng et al., 2016). Furthermore, PICK1 has a role in glutamate receptor regulation (Perroy et al., 2002), and a recent study revealed that a glutamate gene-set showed association with the severity of hyperactivity/impulsivity in an ADHD case-only sample (Naaijen et al., 2017). In addition, adult *Pick1* knockout mice show several behavioral abnormalities, such as hyperactivity and electrophysiological deficits in the prefrontal cortex (Nomura et al., 2016). With the prominent involvement of dopamine regulation in ADHD, as e.g. the dopaminergic system plays an important role in planning and initiation of motor responses, activation, switching, reaction to novelty, and reward processing (Faraone et al., 2015), these molecular findings suggest a link between genetic variation in *AAED1*, dopaminergic and glutamatergic signaling, and ADHD risk. Thus, studies of the *AAED1* variant's functional impact in carrier-derived neurons of dopaminergic and glutamatergic specification, which have been differentiated from induced pluripotent stem cells (iPSCs), are currently being conducted (E. Svirin & K.P. Lesch, unpublished results).

Our combined approach of linkage and WES also identified a rare genetic variant in the *ATAD2* gene, coding for the ATPase family AAA domain-containing protein 2, and gene-based analysis of this gene revealed suggestive association with persistent ADHD. However, a neuronal function of this gene has not been described yet.

The findings described here need to be interpreted in light of several strengths and limitations. Although we considered only three families, we identified *AAED1* as a novel ADHD candidate gene, showing that combining linkage analysis and WES can be an efficient strategy to prioritize ADHD-associated genes/variants. In contrast to previous studies focusing on pre-defined gene-sets (Demontis et al., 2016; Hawi et al., 2016), we performed an exome-wide search for rare variants. Additionally, we validated the association of the newly identified ADHD risk genes in an independent sample. However, two main types of

genetic variation, which may have helped us to find contributing genes in families P1 and P3, remained unstudied. Firstly, genetic variation located in intronic and intergenic regions may be discovered by using whole-genome sequencing approaches. Alternatively, common variants in regulatory regions close to the genes of interest may be imputed and then included in association analyses. Since we know from studies in other psychiatric disorders that many risk variants are located within regulatory regions (Roussos et al., 2014), genetic variation in those regions probably also contributes to the genetic architecture of ADHD. Secondly, CNVs could play a role in the etiology of ADHD, since prior studies have noted an enrichment of large CNVs in ADHD cases (Elia et al., 2010; Lesch et al., 2011; Stergiakouli et al., 2012; Williams et al., 2012), particularly in genes related to neurodevelopment (Williams et al., 2010). Moreover, future studies may aim to integrate data from both rare variants and the common polygenic load in those families, in order to obtain a more complete picture on the genetic architecture of ADHD in the individual families.

In conclusion, we provide evidence for the role of rare variants in protein-coding genes in the etiology of ADHD. Our data adds to the notion that less frequent variants provide an additional source of relevant genetic risk factors, which received little attention in ADHD genetics so far. Moreover, we show that genes harboring rare genetic variants in individual families are associated with persistent ADHD in an independent sample. Therefore, this study suggests that the combination of linkage analyses and WES provides a practical approach for gene identification in genetically complex neurodevelopmental disorders, such as ADHD.

Acknowledgements

The authors wish to thank all participants who took part in the study. We are grateful to S. Gross and M. Dona for the assistance in the recruitment and phenotypic characterization of families and to N. Schraud and G. Ortega for their technical expertise.

This study is part of the International Multicentre persistent ADHD Collaboration (IMpACT; www.impactadhdgenomics.com). IMpACT unites major research centers working on the genetics of ADHD persistence across the lifespan and has participants in the Netherlands, Germany, Spain, Norway, The United Kingdom, The United States, Brazil, and Sweden. Principal investigators of IMpACT are: Barbara Franke (chair), Andreas Reif (co-chair), Stephen V. Faraone, Jan Haavik, Bru Cormand, Antoni Ramos-Quiroga, Philip Asherson, Klaus-Peter Lesch, Jonna Kuntsi, Claiton H. D. Bau, Jan Buitelaar, Stefan Johansson, Henrik Larsson, Alysa Doyle, and Eugenio H. Grevet. This work was partly carried out on the Dutch national e-infrastructure with the support of the SURF Foundation.

Barbara Franke and her team are supported by funding from a personal Vici grant of the Netherlands Organisation for Scientific Research (NWO; grant 016-130-669, to BF), from the European Community's Seventh Framework Programme (FP7/2007 – 2013) under grant agreements n° 602805 (Aggressotype), n° 602450 (IMAGEMEND), and n° 278948 (TACTICS), and from the European Community's Horizon 2020 Programme (H2020/2014 – 2020) under grant agreements n° 643051 (MiND) and n° 667302 (CoCA). In addition, this work was supported by the European College of Neuropsychopharmacology (ECNP Network "ADHD across the Lifespan").

Klaus-Peter Lesch and his team are supported by the Deutsche Forschungsgemeinschaft (DFG: CRU 125, CRC TRR 58 A1/A5), European Community (EC: AGGRESSOTYPE FP7/ No. 602805; Fritz Thyssen Foundation (No. 10.13.1185), ERA-Net NEURON/RESPOND, No. 01EW1602B, and 5-100 Russian Academic Excellence Project.

The Nijmegen Biomedical Study is a population-based survey conducted at the Department for Health Evidence and the Department of Laboratory Medicine of the Radboud university medical center. Principal investigators of the Nijmegen Biomedical Study are L.A.L.M. Kiemeney, A.L.M. Verbeek, D.W. Swinkels and B. Franke. The NBS exome chip data was generated in a research project that was financially supported by BBMRI-NL, a Research Infrastructure financed by the Dutch government (NWO 184.021.007).

The Heinz Nixdorf Recall (HNR) study is supported by the Heinz Nixdorf Foundation (Germany). Additionally, the study is funded by the German Ministry of Education and Science and the German Research Council (DFG; Project SI 236/8-1, SI236/9-1, ER 155/6-1). We are extremely grateful to all investigators who contributed to the generation of this dataset.

Bru Cormand is supported by the Spanish 'Ministerio de Economía y Competitividad' (SAF2015-68341-R) and by AGAUR, 'Generalitat de Catalunya' (2014SGR932).

Marta Ribasés is a recipient of a Miguel de Servet contract from the Instituto de Salud Carlos III, Ministerio de Economía, Industria y Competitividad, Spain (CP09/00119 and CPII15/00023). Her work is also supported by Instituto de Salud Carlos III (PI12/01139, PI14/01700, PI15/01789, PI16/01505), and cofinanced by the European Regional Development Fund (ERDF), Agència de Gestió d'Ajuts Universitaris i de Recerca-AGAUR, Generalitat de Catalunya (2014SGR1357), Departament de Salut, Generalitat de Catalunya, Spain, and a NARSAD Young Investigator Grant from the Brain & Behavior Research Foundation.

Jan Haavik and his team are supported by the K.G. Jebsen Foundation for Medical Research, University of Bergen, the Western Norwegian Health Authorities (Helse Vest).

The Nord-Trøndelag Health Study (The HUNT Study) is a collaboration between the HUNT Research Centre (Faculty of Medicine, Norwegian University of Science and Technology, NTNU), the Nord-Trøndelag County Council, the Central Norway Health Authority and the Norwegian Institute of Public Health.

Sven Cichon is supported by the German Federal Ministry of Education and Research (BMBF) through the Integrated Network IntegraMent (Integrated Understanding of Causes

and Mechanisms in Mental Disorders) under the auspices of the e:Med Program (grant 01ZX1314A). He also receives support by the Swiss National Science Foundation (project no. 310030_156791).

Conflict of interest

BF received educational speaking fees from Merz and Shire. KPL served as a speaker for Eli Lilly and received research support from Medice, as did AR, all outside the submitted work. JH received speaker fees from Eli Lilly, HB Pharma, and Shire. In the past 3 years, JKB has been a consultant to/member of advisory board of/and/or speaker for Janssen Cilag BV, Eli Lilly, Lundbeck, Shire, Roche, Medice, Novartis, and Servier. He has received research support from Roche and Vifor. He is not an employee of any of these companies, and not a stock shareholder of any of these companies. He has no other financial or material support, including expert testimony, patents, royalties. All other authors report no biomedical financial interests or potential conflicts of interest.

Author contributions

The study was initiated and directed by KPL and BF. MRo, JG and CPJ ascertained and clinically characterized the families. JC, MK, and TZ conducted the statistical analyses. KN carried out the exome sequencing, and MP and GP provided bioinformatics input. Analysis of family data, genotyping validations and downstream data analyses were performed by KPL, AR, CJ, OR, GCZ, HW, and co-workers. BF, KPL, and AAV made substantial contribution to the analysis and interpretation of data. JC and MK wrote the first draft of the manuscript with critical input from BF and KPL. TEG, LK, JB, JH, SJ, JARQ, MRi, BC, AR, ES, KPL, BF, JB, KH, ME, and SC contributed data. BF and KPL provided funding for the project. All co-authors provided critical feedback on the manuscript and approved the final version of the manuscript.

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Supplementary methods

Exome-chip sample

Subjects

The exome-chip sample has been described previously (Zayats et al., 2016). All adult ADHD patients examined in the original study (Zayats et al., 2016) were volunteers enrolled through the International Multicenter persistent ADHD CollaboraTion (IMpACT; http://www.impactadhdgenomics.com/). All patients were diagnosed with ADHD according to DSM-IV criteria, i.e. ADHD symptoms were present before 7 years of age. Controls were recruited either at an IMpACT site (Germany, The Netherlands, UiB Norway, and Spain) or through population studies (Germany, The Netherlands, and Norway [The Nord-Trøndelag Health Study; The HUNT study]). All subjects were of European descent which was proven by means of self-report, together with multi-dimensional scaling (MDS; reported in the original publication; (Zayats et al., 2016)). All participants provided signed informed consent in accordance with the Declaration of Helsinki. The study was approved by ethics committees in each collaborating country at the corresponding recruitment center (for details see (Zayats et al., 2016)). Discovery samples were available from four European IMpACT sites: Germany, The Netherlands, Norway, and Spain. Additional Dutch controls for the discovery analyses were derived from the Nijmegen Biomedical Study (NBS, www.nijmegenbiomedischestudie.nl), a population-based survey conducted by the Departments of Epidemiology & Biostatistics and Clinical Chemistry of the Radboud University Medical Center (Galesloot et al., 2017). Approval to conduct the study was obtained from the Institutional Review Board. Controls were unselected for ADHD symptoms. A detailed description of all samples is provided in the Supplementary Appendix 1 of the original publication (Zayats et al., 2016). The replication sample was not utilized in this study. The gene-sets were analyzed in the discovery sample only.

Genotyping, genotype calling and quality control

All subjects of the discovery stage were genotyped on the InfiniumHumanExome array (Illumina, San Diego, CA, USA). The German cases, the full Dutch sample, and the full Spanish sample were genotyped on HumanExome-12v1-1_A; the German controls and the HUNT Norwegian sample of controls were genotyped on HumanExome-12v1_A; and the UiB Norwegian sample was genotyped on HumanExome-12v1_B version of the chip. All genotypes were processed using Illumina GenomeStudio V2011.1 software, with additional genotype assignments implemented in zCall (Goldstein et al., 2012). Best practice guidelines were used to perform quality control (QC) of genotype calls in GenomeStudio (Grove et al., 2013). Further QC was carried out on all data sets using PLINK (Purcell et al., 2007), with the following steps: (1) genotyping rate threshold was set to 98% both for individuals and for SNVs, (2) Hardy–Weinberg test threshold was set to $P<1.00x10^{-05}$, (3) overall heterozygosity

of individuals was screened based on common (minor allele frequency (MAF)⩾1%) and rare (MAF<1%) SNVs separately, with outliers defined as those outside the range of mean ± 3SD of the total heterozygosity observed in a sample, (4) relatedness (PI_HAT) threshold was set to 10% and (5) ethnic homogeneity was guaranteed by means of MDS with HapMap3 populations. Genotype calling and all QC steps were performed for each data set individually. Those samples that were collected in the same country (namely German cases and German controls, Dutch cases and Dutch controls, Norwegian UiB and HUNT samples) were merged using PLINK and additional QC steps were implemented. Specifically, the screening for heterozygosity, cryptic relatedness, and population outliers was performed once more as described above. Finally, four data sets were produced: a combined German sample, a combined Dutch sample, a combined Norwegian sample, and a Spanish sample.

Statistical analyses

Statistical analyses were divided into two main stages: (1) examination of rare coding SNVs (MAF < 1%) and (2) examination of common SNVs (MAF ≥ 1%) with replication in and independent sample.

Prior to the analyses, each subject's genetic substructure characteristics were estimated with principal components analysis implemented in EIGENSTRAT software for each data set individually (Price et al., 2006). Calculation of principal components was performed based only on individuals and SNVs revealing high genotyping rate (⩾99%) and common variants (MAF⩾ 1%) after removal of strand ambiguous SNVs and those in high LD (r2<0.2). Long stretches of LD were also removed prior to calculation of principal components (Price et al., 2008). Rare variants were defined as those with MAF < 1%. The variants were combined per gene and tested for association with adult ADHD in RAREMETAL (Feng et al., 2014). Common SNVs were defined as those with MAF ≥ 1%. The variants were tested using PLINK(Purcell et al., 2007), assuming an additive model.

Gene-based and gene-set analyses in the exome-chip dataset

Statistical analyses were performed using the Multi-marker Analysis of GenoMic Annotation (MAGMA) software package (version 1.02; http://ctglab.nl/software/magma (de Leeuw et al., 2015)). The subjects' genetic substructure was taken into account by including principal components, reflecting their genetic characteristics, as covariates (Zayats et al., 2016). Genome-wide SNP data from a reference panel (1000 Genomes, v3 phase1 (Genomes Project et al., 2010)) was annotated to NCBI Build 37.3 gene locations. This gene annotation file was used to map the exome chip data from the different samples to assign genetic variants to genes, followed by the calculation of gene-based p-values for each sample. For this, a degree of association was calculated for each gene based on both common and rare variants by using principal component regression. Rare variants were defined as those with MAF < 1%, and a burden score was generated for each gene, computed as weighted sum of all rare

variants in that gene. Burden scores are implemented as a pre-processing step. A threshold is first specified on either MAF or MAC to designate SNPs as 'rare'. For each gene, one or more burden scores are then created by summing the rare variants in that gene. The individual rare variants are then removed from the gene and replaced by these burden scores. Analysis of that gene then proceeds as normal, using the chosen gene analysis model (specified by --gene-model) to analyze the burden scores and remaining common variants (if any). No variants in *ANP32B* were observed in the IMpACT exome-chip data and, therefore, only 24 genes were analyzed. For gene-based analyses, single variant p-values within a gene were transformed into a gene-statistic by taking the mean of the χ2-statistic among variants in each gene. To account for LD, the 1000 Genomes Project European sample was used as a reference to estimate the LD between variants within (the vicinity of) genes (http://ctglab.nl/software/MAGMA/ref_data/g1000_ceu.zip). Gene-wide p-values were converted to z-values reflecting the strength of the association of each gene with ADHD risk, with higher z-values corresponding to stronger associations. Subsequently, we tested whether all 24 genes in the overall gene-set were jointly associated with persistent ADHD, using an intercept-only linear regression model including a subvector corresponding to the genes in the gene-set. This self-contained analysis evaluates whether the regression coefficient of this regression was ≥0, testing whether the overall gene-set showed association with persistent ADHD. Next, we tested whether genes in this gene-set were more strongly associated with persistent ADHD than others genes in the genome. With this competitive test, differences between the association of the gene-set to genes outside this gene-set is tested, accounting for the polygenic nature of a complex disorder like ADHD. Potential confounders, such as gene size and gene density, were included in the competitive test. Because of this, we were more interested in the competitive than the self-contained test for the current analyses.

Supplementary tables

Supplementary Table 1. Candidate linkage regions selected for further analysis containing a maximum logarithm of the odds (LOD) score higher than 2 in at least one of analysis performed.

Chr	Start marker	End marker	Region	Max. LOD score	Analysis
6	rs9392298	rs1113387	203878-460901	3.003	Pedigree 3
6	rs6596970	rs12211028	3446942-4470581	3.003	Pedigree 3
8	rs1993979	rs11776334	118608158-124649389	2.2030	Pedigree 1 and 2 combined
9	rs10758897	rs7875367	7754113-15568230	3.6246	Pedigree 1 and 2 combined
9	rs7847004	rs7847405	97466973-102213749	2.0735	All pedigrees combined
10	rs10906640	rs17395983	14311273-15844850	2.3948	Pedigree 3
10	rs1612430	rs7919353	56177098-58789387	2.5803	Pedigree 2
10	rs10509177	rs2601705	64668048-65875491	2.713	Pedigree 2 and 3 combined
11	rs324256	rs4923591	21968768-29134515	2.0918	Pedigree 2
11	rs4938200	rs12364480	115218677-120365028	3.3631	Pedigree 1 and 3 combined
13	rs9558670	rs16972472	106701406-109091885	2.5168	Pedigree 2 and 3 combined
16	rs153331	rs7198762	63079319-66386711	3.9898	Pedigree 1 and 3 combined
16	rs7200569	rs11150541	81159781-83154022	3.7949	Pedigree 1 and 3 combined

Supplementary Table 2. WES sequencing statistics per individual.

Family	ID	bp sequenced	bp mapped on-target	Total number reads	Total mapped reads on-target	Percentage of on-target reads (%)	Mean Coverage
1	1	4432495337	3900102767	91460024	79681085	87.12	65.08
1	2	5012989993	4411768262	103829553	90405567	87.07	73.31
1	4	4185095550	3543760472	86630343	72553451	83.75	57.42
1	5	4769484984	4217957342	99420668	87006501	87.51	71.26
1	11	4453320723	3911917639	92094198	80066716	86.94	64.52
2	21	6455493150	5851516254	135431851	121571589	89.77	106.42
2	26	7308540150	6672649219	153224671	138653596	90.49	121.56
3	17	6270779135	5667764511	133028891	118744178	89.26	103.01
3	19	4931660430	4303489528	101845716	87873677	86.28	72.42
3	20	6818067496	6191537997	144434109	129623720	89.75	112.32

Supplementary Table 3. List of rare variants identified using WES within linkage regions. In total, we identified 38 rare variants, of which 32 were unique and located within a gene.

Family	Variant ID	rs ID	Gene name	Gene component	Type
1	chr9:12708958->A	------	*TYRP1*	Splice site	-------
1	chr9:14775859G>A	rs10733289	*FREM1*	Exon	Synonymous
1	chr11:114421857T>C	rs78453693	*NXPE1*	Intron	------
1	chr11:114421861G>A	rs76118887	*NXPE1*	Intron	------
1	chr11:114421895G>A	rs78257939	*NXPE1*	Intron	------
1	chr11:114421927G>A	rs78842965	*NXPE1*	Intron	------
1	chr11:114421962G>A	rs75409523	*NXPE1*	Intron	------
1	chr11:118405343G>A	rs512849	*TMEM25*	Intron	------
1	chr11:118927013->C	rs75923954	*HYOU1*	Intron	------
1	chr11:118939939->C	rs199535207	*VPS11*	Exon	Non-synonymous
1	chr11:119031841->C	------	*ABCG4*	UTR	------
1	chr11:119065484->C	------	*CCDC153*	Intron	------
1	chr16:67197776->G	------	*HSF4*	Intron	------
1	chr16:80581631G>A	rs6564764	*DYNLRB2*	Intron	------
1	chr16:81145675T>C	rs8059153	*PKD1L2*	Intron	------
1	chr16:81242198G>A	rs7499011	*PKD1L2*	Exon	Non-synonymous
1	chr16:81816787T>A	rs4476171	*PLCG2*	Intron	------
1	chr16:83992785G>A	rs2665296	*OSGIN1*	Intron	------
1	chr16:84132628->AC	rs202131529	*MBTPS1*	Intron	------
2	chr8:120940874T>C	rs62528677	*DEPTOR*	Intron	------
2	chr8:124346225T>C	------	*ATAD2*	Exon	Non-synonymous
2	chr9:9425588->ACTT	rs200022326	*PTPRD*	Intron	------
2	chr9:12694274G>A	rs61752937	*TYRP1*	Exon	Non-synonymous
2	chr9:14775859G>A	rs10733289	*FREM1*	Exon	Synonymous
2	chr9:99064202->A	------	*HSD17B3*	Intron	------
2	chr9:99404124G>C	rs151326868	*AAED1*	Exon	Non-synonymous
2	chr9:100756891->T	------	*ANP32B*	Intron	------
2	chr9:100995758G>T	rs879368	*TBC1D2*	Exon	Non-synonymous
2	chr10:55590946CAGAC>T	------	*PCDH15*	Intron	------
2	chr11:26574783G>-	rs143835466	*ANO3*	Splice site	------
2	chr11:116633913A>G	------	*BUD13*	Exon	Non-synonymous
2	chr11:118939939->C	rs199535207	*VPS11*	Exon	Non-synonymous
3	chr11:116633913A>G	------	*BUD13*	Exon	Non-synonymous
3	chr11:118405343G>A	rs512849	*TMEM25*	Intron	------
3	chr11:118939939->C	rs199535207	*VPS11*	Exon	Non-synonymous
3	chr16:66432304ACCACCCC>-	rs113303884	*CDH5*	Intron	------
3	chr16:84132628->AC	rs202131529	*MBTPS1*	Intron	------

Supplementary Table 4. Results of the gene-set association analyses of the individual IMpACT exome-chip samples (Zayats et al., 2016).

Cohort	N (cases/controls)	Gene-set	N genes	$P_{self\text{-}contained}$	$P_{competitive}$
Spain	(615/932)	General	23	0.074232	0.034095
		P1	13*	0.8862	0.8772
		P2	12	0.00067007	0.00013433
		P3	5	0.10719	0.026187
Norway	(597/2,598)	General	23	0.038002	0.055531
		P1	13*	0.038847	0.05538
		P2	12	0.051919	0.078343
		P3	5	0.67716	0.65549
Germany	(340/2,286)	General	24	0.21395	0.30365
		P1	14	0.38634	0.50658
		P2	12	0.50793	0.53963
		P3	5	0.074312	0.18876
Netherlands	(294/1,703)	General	24	0.16794	0.11289
		P1	14	0.63434	0.66457
		P2	12	0.30359	0.16463
		P3	5	0.41871	0.41045

*No variants were observed in the *DYNLRB2* gene in this sample of the IMpACT exome-chip data.

Supplementary Table 5. Minor allele frequencies and summary statistics for common variants from meta-analysis of the exome-chip data.

							SPAIN		NORWAY		GERMANY		NETHERLANDS		SUMMARY STATISTICS FOR COMMON VARIANTS FROM META-ANALYSIS (Beta is reported for Allele1)	
Name	**Chr**	**MapInfo**	**rs-number**	**Gene(s)**	**Allele 1**	**Allele 2**	**MAF-cases**	**MAF-controls**	**MAF-cases**	**MAF-controls**	**MAF-cases**	**MAF-controls**	**MAF-cases**	**MAF-controls**	**Beta**	**…**
exm764637	9	99404115	rs142681714	AAED1 (C9orf21)	G	A	0	0.001609	0	0	0	0.0004374	-	-	-	…
exm764638	9	99404124	rs151326868	AAED1 (C9orf21)	C	G	0	0.001609	0	0	0.004412	0.0004374	0	0.001468	-	…
exm764639	9	99404131	-	AAED1 (C9orf21)	T	G	-	-	0.0008375	0	0	0.0008749	0.003401	0.0005872	-	…
exm764646	9	99408211	-	AAED1 (C9orf21)	C	G	0	0.0005365	0.0008375	0	0	0.0002187	0.001701	0	-	…
exm764655	9	99413728	-	AAED1 (C9orf21)	C	T	0.000813	0	-	-	-	-	-	-	-	…
exm764662	9	99417000	rs9886834	AAED1 (C9orf21)	A	G	0.001626	0.005901	-	-	0.001471	0	0	0.0008808	-	…
exm962101	11	119029041	-	ABCG4	T	C	-	-	-	-	-	-	0	0.0002936	-	…
exm962077	11	119025514	rs141832911	ABCG4	A	G	-	-	0	0.001155	-	-	-	-	-	…
exm2249608	11	119030981	-	ABCG4	A	G	-	-	0.0008375	0	-	-	-	-	-	…
exm962097	11	119027724	rs139879498	ABCG4	A	G	0	0.0005365	0.0008375	0	0	0.0008749	0	0.0002936	-	…
exm1697301	11	119027691	rs12271907	ABCG4	G	C	0.002439	0.003755	-	-	0	0.0006562	-	-	-	…
……	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…

*The complete **Supplementary Table 5** is available upon request.*

Supplementary Table 6. Count data for all markers of the exome-chip data is shown per site.

Variant	CHR	BP	genotypes	counts_in_controls	counts_in_cases
exm717719	8	120940733	CC	2597	597
			GC	1	0
exm717740	8	120977489	CC	2	1
			CT	194	38
			TT	2402	558
exm717741	8	120977542	CT	44	20
			TT	2554	577
exm717755	8	121013770	CC	1410	338
			TC	1017	218
			TT	171	41
exm717770	8	121015349	GG	2595	597
			TG	3	0
exm717787	8	121061879	AA	279	62
			AG	1132	265
			GG	1187	270
exm718925	8	124335225	AA	2563	585
			GA	35	12
exm718965	8	124348685	AA	2598	596
			GA	0	1
exm718971	8	124351667	CC	2595	596
			TC	3	1
exm718973	8	124357252	CC	2593	595
			TC	5	2
exm719045	8	124382144	CT	0	1
			TT	2598	596
exm2248310	9	8319965	CC	2598	596
			TC	0	1
exm738865	9	8331667	CC	2596	597
…	…	…	…	…	…
			…	…	…

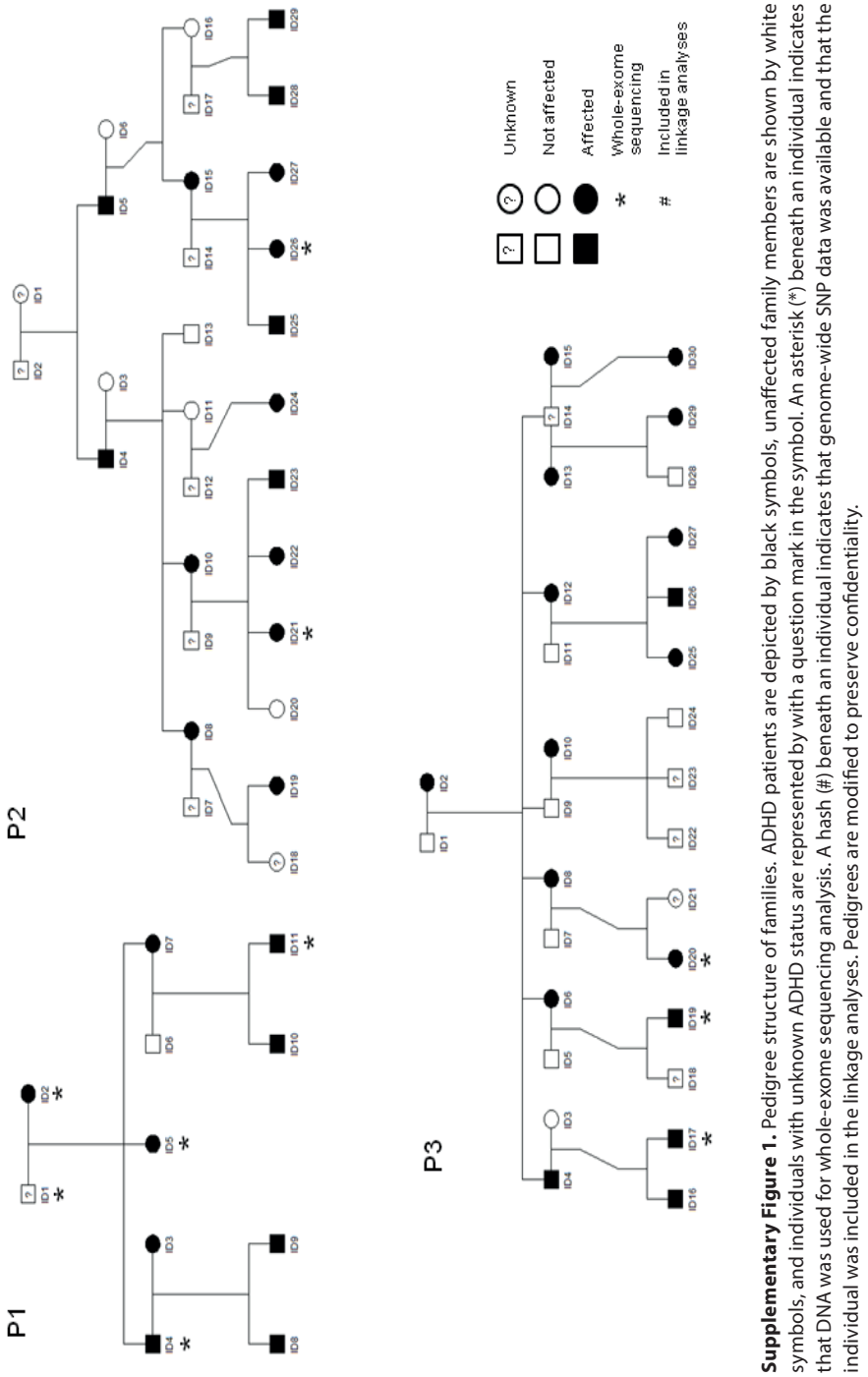
Tab 1=Norway, tab 2=Spain, tab 3=Germany, tab 4=Netherlands

*The complete **Supplementary Table 6** is available upon request.*

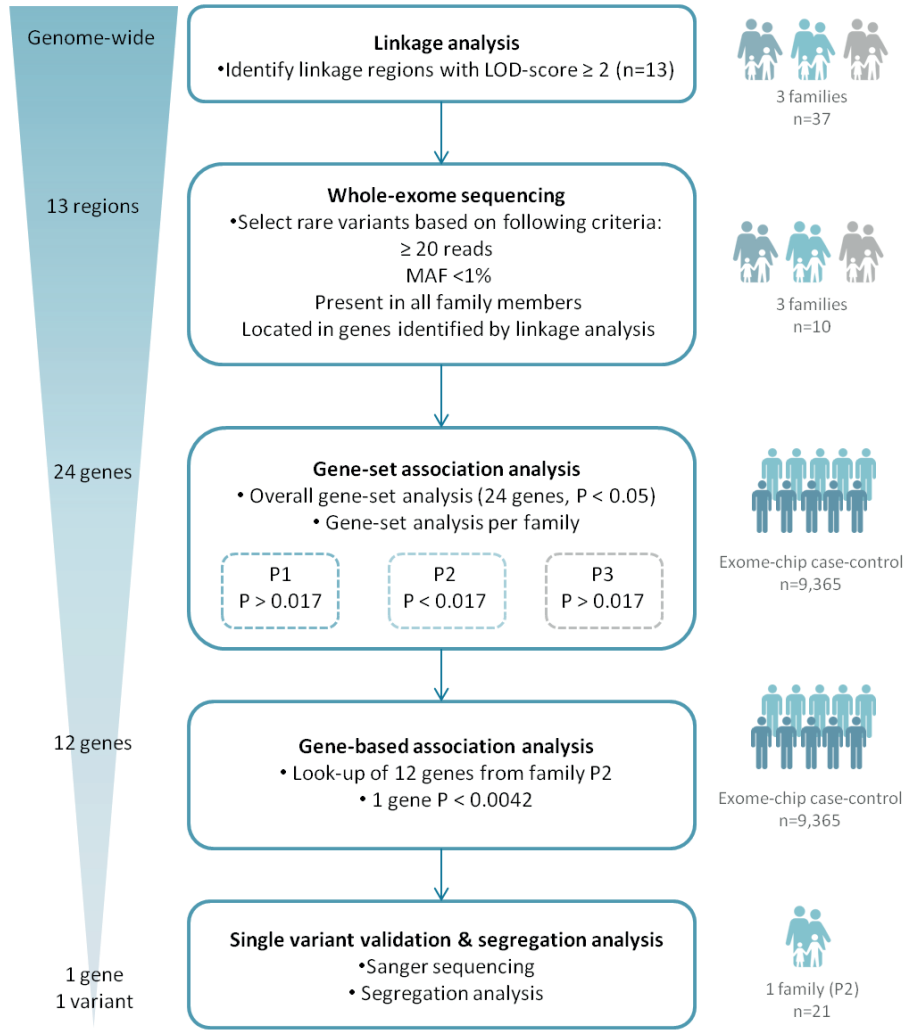
Supplementary Table 7. Information on two rare variants from family P2 that were selected for segregation analysis.

Gene	Variant ID	Frequency ExAC	Variant type	Amino acid change	Gene component	phyloP	Grantham Score	Polyphen2	SIFT score	MutationTaster
AAED1	chr9:99404124G>C rs151326868	0.0004375	Substitution – non-synonymous	H200D	EXON	5.213	81	0.999 (probably damaging)	0.04 (deleterious)	P=1 (disease causing)
ATAD2	chr8:124346225T>C	0.0000082	Substitution – non-synonymous	H1124R	EXON	2.06	29	0.048 (benign)	0.13 (tolerated)	P=0.903 (disease causing)

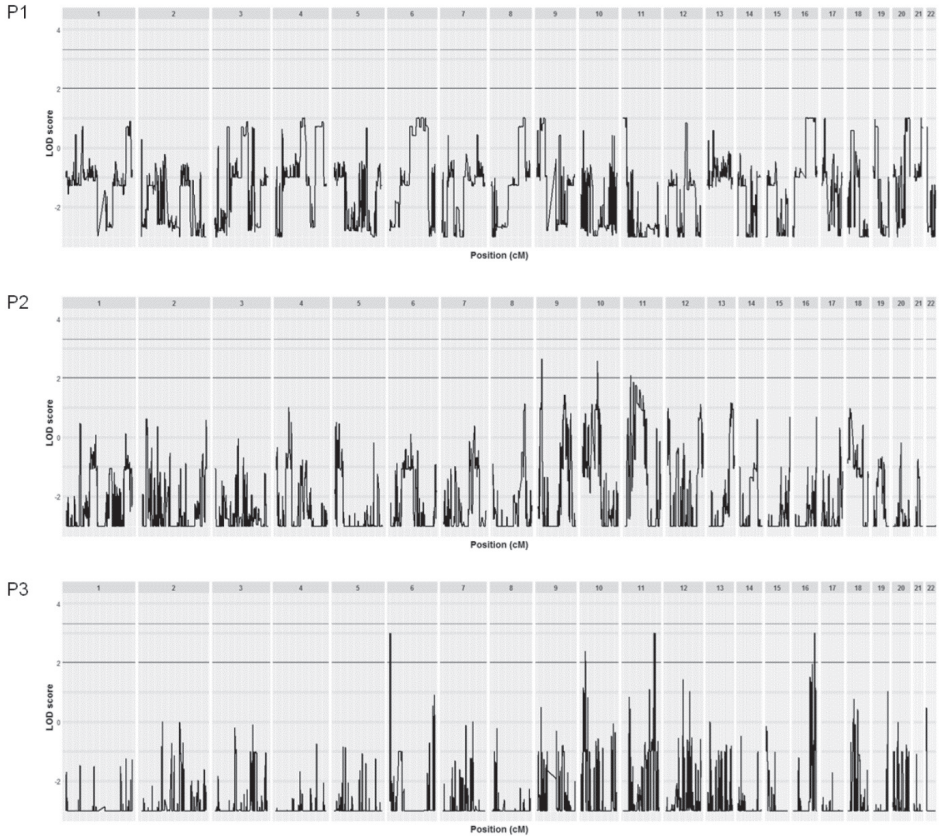
Supplementary Figures



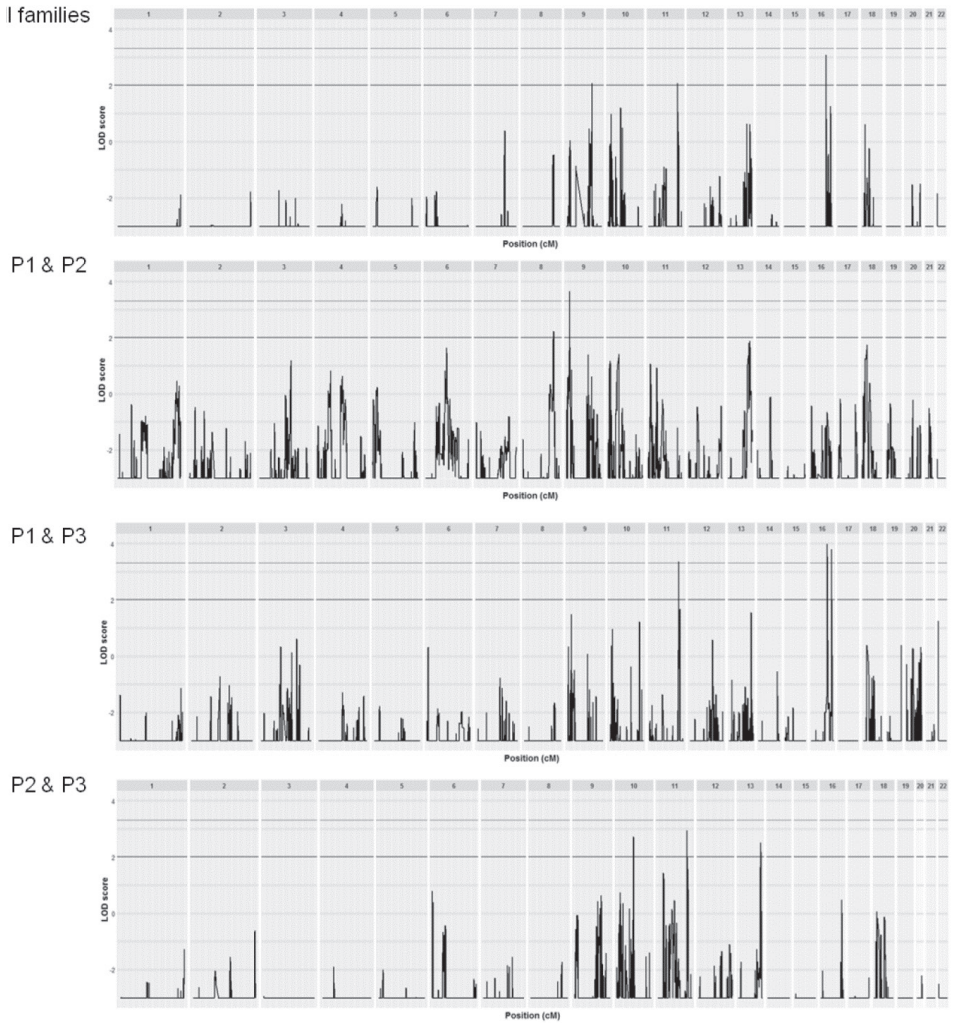
Supplementary Figure 1. Pedigree structure of families. ADHD patients are depicted by black symbols, unaffected family members are shown by white symbols, and individuals with unknown ADHD status are represented by with a question mark in the symbol. An asterisk (*) beneath an individual indicates that DNA was used for whole-exome sequencing analysis. A hash (#) beneath an individual indicates that genome-wide SNP data was available and that the individual was included in the linkage analyses. Pedigrees are modified to preserve confidentiality.



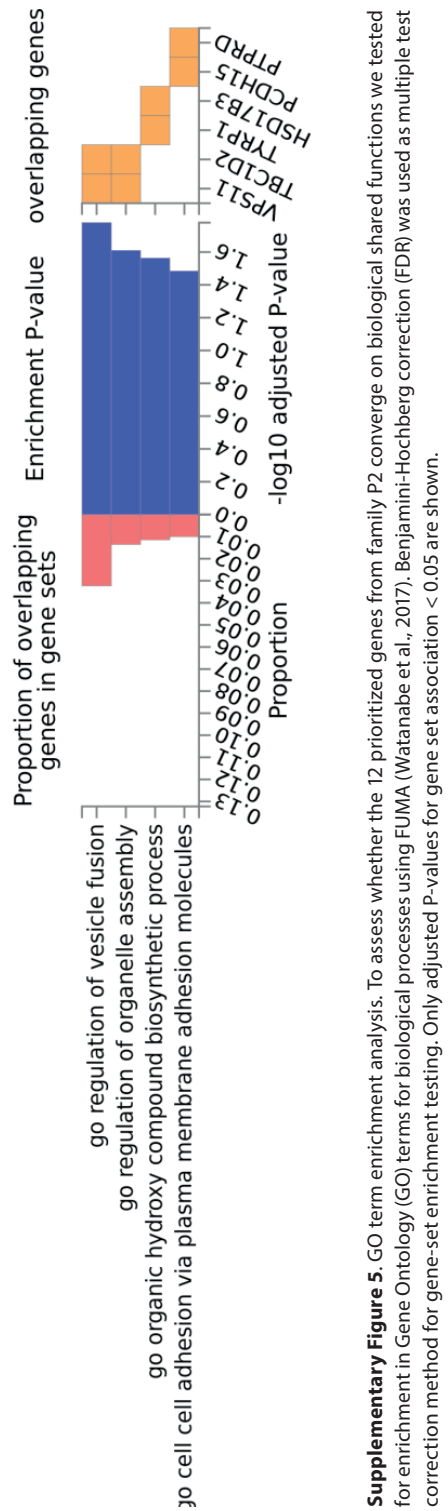
Supplementary Figure 2. Schematic overview of the study design and statistical approach. Our study included five main steps for the identification of novel ADHD genes. By combining linkage analysis and whole-exome sequencing (WES) in three multigeneration pedigrees with multiple affected individuals, we prioritized those genes emerging from linkage regions and harboring rare variants. Those 24 genes were taken forward and were jointly analyzed in gene-set analyses of IMpACT exome-chip data. Subsequently, family-wise gene-set analysis was performed, and 12 genes from family P2 were taken forward for gene-based analyses. One gene showed significant gene-based association, and a rare variant was validated.



Supplementary Figure 3. Genome-wide logarithm of the odds (LOD) score graph for families P1, P2, and P3 individually. The lower horizontal, black line represents a LOD score of 2 and the upper horizontal, grey line indicates the significance level at a LOD score of 3.3.



Supplementary Figure 4. Genome-wide logarithm of the odds (LOD) score graph for the different combinations of the three families. The lower horizontal, black line represents a LOD score of 2 and the upper horizontal, grey line indicates the significance level at a LOD score of 3.3.



Supplementary Figure 5. GO term enrichment analysis. To assess whether the 12 prioritized genes from family P2 converge on biological shared functions we tested for enrichment in Gene Ontology (GO) terms for biological processes using FUMA (Watanabe et al., 2017). Benjamini-Hochberg correction (FDR) was used as multiple test correction method for gene-set enrichment testing. Only adjusted P-values for gene set association < 0.05 are shown.

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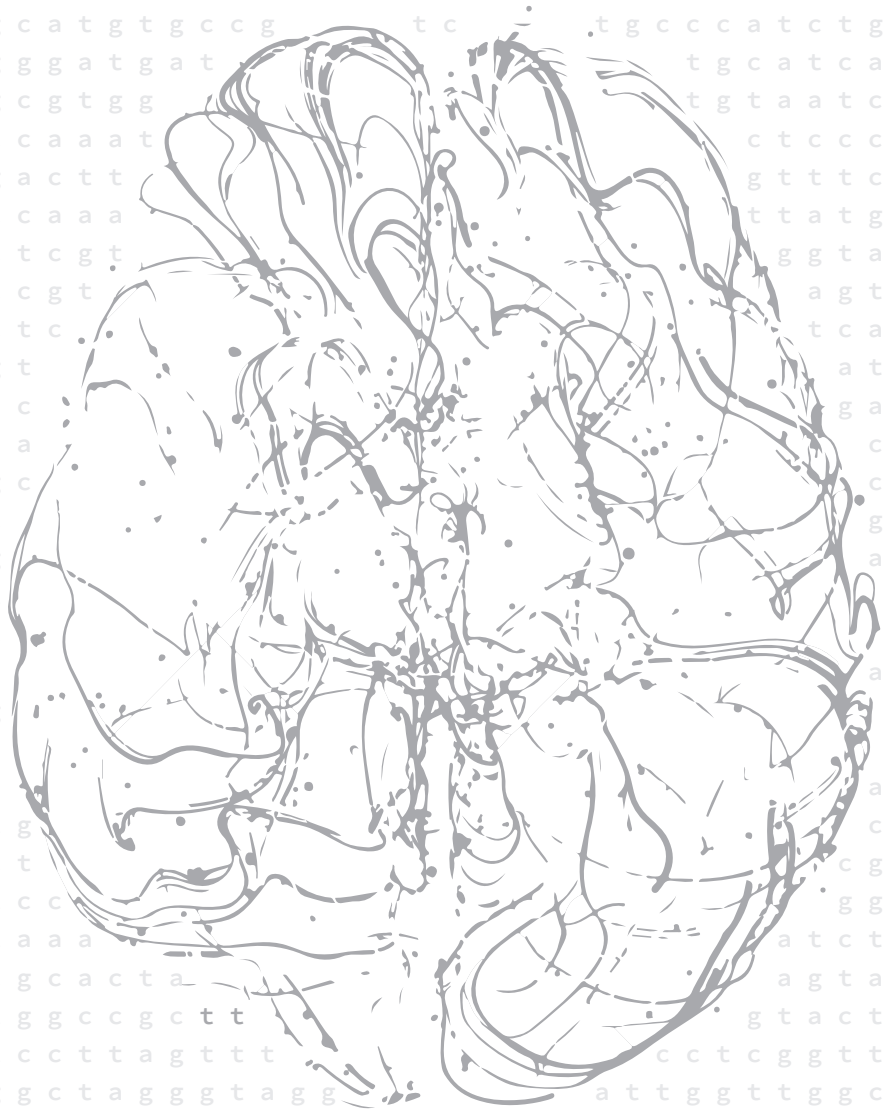
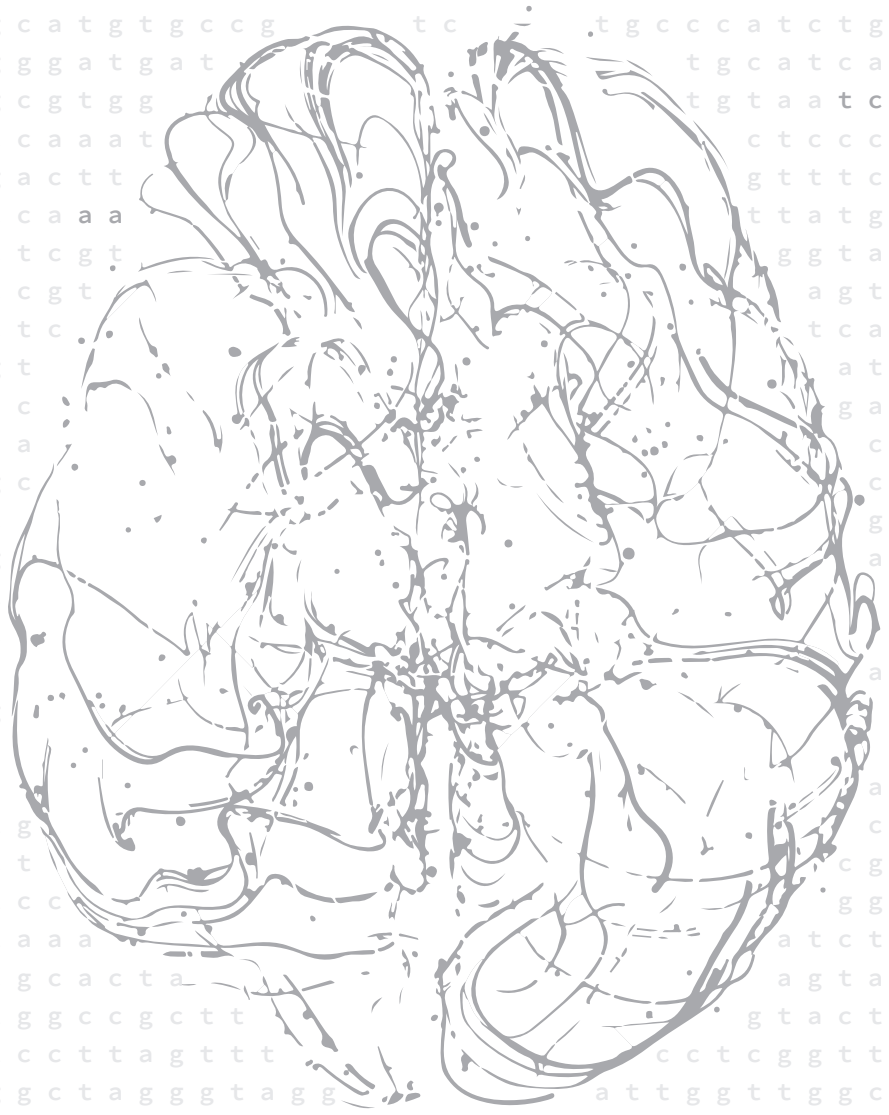


PART 2

Mapping of mechanisms from gene to disorder



CHAPTER 7

Imaging genetics in neurodevelopmental psychopathology

Published as:
M. Klein*, M. M. van Donkelaar*, E. Verhoef, and B. Franke (2017). "Imaging genetics in neurodevelopmental psychopathology." American Journal of Medical Genetics Part B: Neuropsychiatric Genetics **174**(5): 485-537.
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Abstract

Neurodevelopmental disorders are defined by highly heritable problems during development and brain growth. Attention-deficit/hyperactivity disorder (ADHD), autism spectrum disorders (ASDs), and intellectual disability (ID) are frequent neurodevelopmental disorders, with common comorbidity among them. Imaging genetics studies on the role of disease-linked genetic variants on brain structure and function have been performed to unravel the etiology of these disorders. Here, we reviewed imaging genetics literature on these disorders attempting to understand the mechanisms of individual disorders and their clinical overlap. For ADHD and ASD, we selected replicated candidate genes implicated through common genetic variants. For ID, which is mainly caused by rare variants, we included genes for relatively frequent forms of ID occurring comorbid with ADHD or ASD. We reviewed case-control studies and studies of risk variants in healthy individuals. Imaging genetics studies for ADHD were retrieved for *SLC6A3/DAT1*, *DRD2*, *DRD4*, *NOS1*, and *SLC6A4/5HTT*. For ASD, studies on *CNTNAP2*, *MET*, *OXTR*, and *SLC6A4/5HTT* were found. For ID, we reviewed the genes *FMR1*, *TSC1 and TSC2*, *NF1*, and *MECP2*. Alterations in brain volume, activity, and connectivity were observed. Several findings were consistent across studies, implicating e.g. *SLC6A4/5HTT* in brain activation and functional connectivity related to emotion regulation. However, many studies had small sample sizes, and hypothesis-based, brain region-specific studies were common. Results from available studies confirm that imaging genetics can provide insight into the link between genes, disease-related behavior, and the brain. However, the field is still in its early stages, and conclusions about shared mechanisms cannot yet be drawn.

Keywords: ADHD, ASD, ID, brain imaging genetics, neurodevelopmental disorders

Introduction

Neurodevelopmental disorders are broadly defined as disorders in the development and growth of the brain (Goldstein and Reynolds, 1999), but this term is largely used to describe neurological and psychiatric disorders that have their onset prior to adulthood. Most neurodevelopmental disorders are highly heritable, either caused by single genetic defects, like many of the intellectual disability (ID) disorders (Deciphering Developmental Disorders Study, 2015), or with a more multifactorial background, in which several to multiple less penetrant genetic variants cause the disease in combination with environmental factors, like in many cases of autism spectrum disorders (ASDs; (Gaugler et al., 2014; Iossifov et al., 2014), as well as in attention-deficit/hyperactivity disorder (ADHD; (Faraone et al., 2015; Franke et al., 2012), oppositional defiant disorder, and conduct disorder (Salvatore and Dick, 2016).
While technological advances in the last decade, especially genome-wide association studies (GWASs) and next generation sequencing, have enabled the identification of many genetic factors involved, the biological mechanisms contributing to the neurodevelopmental disorders are still largely unknown. It is thought that gene variation/mutation will alter molecular and cellular processes, which leads to altered brain development, be it structurally and/or functionally, and subsequently to altered behavior and disease symptoms (Franke et al., 2009). Measures that mediate the effects of genes on behavioral/disease phenotypes have been termed endophenotypes or intermediate phenotypes (Gottesman and Gould, 2003; Kendler and Neale, 2010).

Much research into the consequences of gene aberrations is performed in animal models. However, brain imaging methods like magnetic resonance imaging (MRI), electroencephalography (EEG), and magnetoencephalography (MEG) offer excellent ways to investigate the effects of genetic variation on brain structure, function, and connectivity directly in humans *in vivo*. Such 'imaging genetics' approaches can unveil the brain-biological consequences of molecular changes induced by genetic variants – both common and rare – linked to neurodevelopmental disorders. In that way they can help to understand the mechanisms through which differences in behavior arise. It has been argued that the effects of disease-linked (common) genetic variation on the brain would be larger than those on behavior and clinical phenotypes (Gottesman and Gould, 2003; Rose and Donohoe, 2013)), although more recent work using hypothesis-free imaging genetics approaches argues against this – at least for brain structural phenotypes (Franke et al., 2016).

Different neuroimaging methods can be used in imaging genetics studies, including different forms of structural and functional MRI as well as EEG and MEG. They have complementary characteristics enabling information to be gathered on different aspects of (gene effects on) brain anatomy and function, like location (especially MRI-based methods) and timing (especially EEG and MEG). In this review, we concentrated on those methods that have most frequently been used in imaging genetics studies of neurodevelopmental

disorders, i.e. MRI-based methods evaluating gene effects on brain structure, function, and connectivity.

With structural magnetic resonance imaging (sMRI) it is possible to noninvasively characterize the structure of the human brain. Thereby, the different magnetic properties of brain tissues are used to map the spatial distribution of these structural properties of the brain. In this way, the different brain tissues (grey and white matter) and cortical and subcortical structures of the brain can be mapped. By adapting scanning parameters, different weighting techniques of the signal can be used, such as T1-weighted imaging (used to visualise anatomy) and T2-weighted imaging (which is useful for demonstrating lesions and pathology). Different aspects of brain structure can be used for quantitative analyses. To investigate whether volumetric differences are global or regional, specific brain regions of interest (ROIs) can be selected *a priori* and studied individually. In contrast, global changes in grey or white matter intensity can be detected by using voxel-based morphometry (VBM) analyses. Next to volumetric differences observed in grey matter, structural differences of white matter connectivity can also be quantified. With the help of diffusion tensor imaging (DTI), it is possible to non-invasively investigate the macrostructural integrity and orientation of white matter fibre bundles. Thereby, the directional diffusion of water molecules along neuronal membranes is measured, allowing to map white matter connection within the brain. Multiple measures can be derived from DTI. A frequently measured parameter is fractional anisotropy (FA). Basically, anisotropy indicates that diffusion takes place in a directional manner, whereas isotropy indicates diffusion in all directions. Additional DTI-derived parameters include mean diffusivity (MD; average of axial diffusivity (AD) and perpendicular diffusivities), and radial diffusivity (RD; average of perpendicular diffusivities), the mode of anisotropy (sensitive to crossing fibres), and the apparent diffusion coefficient (indicating the magnitude of diffusion) (Le Bihan, 2003; Le Bihan et al., 2001; Yoncheva et al., 2016).

Resting state functional MRI (rs-fMRI), allows to analyse the temporal correlations of neural activity across anatomically disparate brain regions and thereby to examine the functional connectivity based on spontaneous brain activity, neural organization, and circuit architecture.

To investigate potential changes in brain activity, functional magnetic resonance imaging (fMRI) can be used. Since fMRI is sensitive to the oxygenation of the blood, the so-called blood-oxygen-level-dependent (BOLD) signal can be measured. Thereby brain function is measured, based on the premise that active cells consume oxygen, thus causing changes in blood oxygenation, and subsequently leading to increased blood flow. However, the exact link between cell activation, oxygen saturation, and cerebral blood flow changes is debatable (Hillman, 2014). Generally in fMRI, alterations in blood flow after e.g. a task-induced stimulus or during a resting condition are measured.

Here, we systematically reviewed the imaging genetics literature for three frequent neurodevelopmental disorders, ADHD, ASDs, and selected intellectual disability (ID) disorders. The choice for those three neurodevelopmental disorders was based on their frequent comorbidity (Vorstman and Ophoff, 2013) and robustly established associations with specific genetic variants. The aim of this work was to extract core brain mechanisms affected by disease-linked genetic factors related to the individual disorders as well as their clinical overlap.

ADHD is one of the most common neurodevelopmental disorders, with a prevalence of 5-6% in childhood (American Psychiatric Association, 2013; Polanczyk et al., 2007). ADHD can be clinically characterized by two core symptom domains: inattention and hyperactivity/impulsivity (American Psychiatric Association, 2013; Faraone et al., 2015). Up to 60% of all patients diagnosed in childhood show ADHD symptoms and/or meet formal diagnostic criteria for the disorder in adulthood, and prevalence rates of persistent ADHD in adults range between 2.5 and 4.9% (Simon et al., 2009). ASD affects approximately 0.6% to 1% of the children, making it one of the most prevalent disorders in childhood (Elsabbagh et al., 2012). Although there are some important differences in core symptom definition, the co-occurrence between ADHD and ASD is supported by clinical (Craig et al., 2015), common biological (Rommelse et al., 2010), and non-biological risk factors (Kroger et al., 2011). Moreover, several studies identified that symptoms of autism or autistic traits appear in 20% to 30% of children with ADHD (Grzadzinski et al., 2011; Kochhar et al., 2011). Additionally, ADHD is a common comorbid disorder in children with ID, and the risk increases with increasing severity of ID (Voigt et al., 2006). Studies of children with mild and borderline ID have identified ADHD in 8% to 39% of the cases (Baker et al., 2010; Dekker and Koot, 2003; Emerson, 2003). ADHD is highly heritable (heritability 70-80%) (Burt, 2009; Faraone et al., 2005). However, identification of ADHD risk genes has been difficult (Franke et al., 2009; Gizer et al., 2009), mainly due to ADHD's complex genetic background (Faraone et al., 2015; Franke et al., 2012). Mostly genetic variants, which occur quite frequent in the population and have generally small effects on disease risk have been investigated for their role in ADHD until today, either through candidate gene studies or hypothesis-free GWASs. Only a few of the candidate genes have been confirmed through meta-analysis (Gizer et al., 2009). However, none of the eleven GWAS (Hinney et al., 2011; Lasky-Su et al., 2008a; Lasky-Su et al., 2008b; Lesch et al., 2008; Mick et al., 2010; Neale et al., 2008; Neale et al., 2010a; Sanchez-Mora et al., 2014; Sonuga-Barke et al., 2008; Stergiakouli et al., 2012; Yang et al., 2013) nor a meta-analysis of many of them (Neale et al., 2010b) published to date, reported any genome-wide significant risk variant.

ASDs refer to a heterogeneous group of neurodevelopmental disorders diagnosed in approximately 1 of 88 children (Autism and Developmental Disabilities Monitoring Network Surveillance Year 2008 Principal Incestigators and Centers for Disease Control and Prevention, 2012). It is characterized by deficits in social behavior and language development, as well as

restricted or stereotypic interests (American Psychiatric Association, 2013). About 70% of individuals with ASDs have some level of ID while the remaining 30% have some disability (speech, behavior) other than cognitive dysfunction (Mefford et al., 2012). Whereas early reports estimated ASD heritability to be higher than 90% (Bailey et al., 1995; Folstein and Rutter, 1977; Ritvo et al., 1985; Steffenburg et al., 1989), recent population-based studies provided an estimate of ~50% heritability (Gaugler et al., 2014; Sandin et al., 2014). ASDs are genetically highly complex, as part of the cases has oligogenic or even monogenic causes (with an important role for *de novo* mutations (Iossifov et al., 2014)), whereas the concerted action of common genetic variants of individually small effect sizes and environmental factors is likely to cause most of the disease burden of ASDs (Iossifov et al., 2014) (Gaugler et al., 2014; Zhao et al., 2007). Several of those common variants contributing to ASD risk have been identified through hypothesis-driven studies. Until now, three GWASs have been performed for ASDs (Anney et al., 2010; Wang et al., 2009; Weiss et al., 2009), which identified a single locus on chromosome 5p14, in-between *CDH10* and *CDH9* (Wang et al., 2009). Association with this locus might be driven by markers located within the *MSNP1AS* pseudogene (Ma et al., 2009).

ID refers to a highly heterogeneous group of disorders characterized by below average intellectual functioning (IQ < 70) in conjunction with significant limitations in adaptive functioning with onset during development. ID may occur as an isolated phenomenon or accompanied with malformations, neurological signs, impairment of the special senses, seizures and behavioral disturbances (van Bokhoven, 2011). ID has an estimated prevalence of approximately 2% to 3%, and approximately 0.3% to 0.5% of the population is severely handicapped (Perou et al., 2013). Comorbidity with ADHD and ASDs is frequently observed (Vorstman and Ophoff, 2013). Disease etiology of ID is thought to be largely monogenic, but with many different genetic anomalies implicated (van Bokhoven, 2011). Genetic causes of ID range from large cytogenetically visible chromosomal aberrations, such as trisomy 21, to translocations, subchromosomal abnormalities (such as Prader-Willi syndrome (15q11.2-q13)), copy number variations, and to single gene defects. We concentrated only on the latter in our review, based on the assumption that we can learn most from understanding effects of specific genes/variants on brain structure, function and connectivity. While in many ID disorders, a defect in a single gene can be identified as the cause of the disorder, only a few genes are hit more frequently and cause relatively common ID disorders. To prevent bias of our review by single case reports, we concentrated on those common forms of ID, especially selecting those, in which comorbidity with ADHD and ASD is common. This resulted in five ID disorders included in this review: fragile X syndrome, tuberous sclerosis, neurofibromatosis type 1, Rett syndrome, and Timothy syndrome. Fragile X syndrome (FXS), caused by genetic defects in the *FMR1* gene, is associated with a variable clinical phenotype, including intellectual disabilities with a broad range of severities. IQ is 40 on average for affected men (Merenstein et al., 1996) and normal or borderline in females (de Vries et al., 1996), who show a milder phenotype because the disorder is X-chromosome-linked. High rates of autism and autistic behaviors are seen in individuals with FXS (Hagerman et al., 2009), and 59% of FXS subjects shows ADHD symptoms (Sullivan et al., 2006). Neurofibromatosis type 1 (NF1), caused by mutations in *NF1*, is associated with the presence of usually benign neurofibromas. While IQ in general is average to low average, up to 8% of children with NF1 have an IQ below 70. Learning difficulties and neuropsychological deficits are common, and the core cognitive impairments are in visual spatial function, attention, executive function, and language skills. About 38% of children with NF1 meet diagnostic criteria for ADHD, and a substantial proportion of subjects show social deficits related to ASD (Hyman et al., 2005; Walsh et al., 2013). Tuberous sclerosis complex (TSC) is caused primarily by mutations in the genes *TSC1* and *TSC2* and is characterized by benign hamartomas in multiple organ systems, including the brain. Intellectual ability in TSC ranges from normal to profoundly impaired, and neurobehavioral abnormalities and epilepsy are common. Both ASD and ADHD are reported in about 50% of individuals with TSC, with an even higher number of diagnoses in intellectually impaired individuals (Prather and de Vries, 2004). Rett syndrome, caused by mutations in the *MECP2* gene, primarily affects females. Language problems and cognitive and motor deficits start to become obvious around the age of 6 months in the patients. Testing of cognitive dysfunction is difficult because of a characteristic absence of speech, but ASD-related features, such as avoidance of eye contact, are common (Armstrong, 2005). Timothy syndrome is a multisystem disorder caused by missense mutations in the *CACNA1C* gene. Neurodevelopmental features include global developmental delays and ASDs. Average age of death is 2.5 years, usually caused by ventricular tachyarrhythmia, infection, or complications of hypoglycemia (Splawski et al., 1993).

With this review, we aimed at providing a comprehensive overview on the imaging genetics literature for the three neurodevelopmental disorders. To prevent bias, we excluded reports including less than 10 cases and focused on specific genetic variants, which for ADHD and ASDs resulted in a focus on genes/loci implicated through variants that are common in the population, and for ID, we restricted the review to the genes causing the single-gene ID disorders described above. While imaging genetics studies have been performed in patients, the underlying candidate genes and their common genetic variants are also frequently studied in healthy individuals. This allows analysis of effects of common genetic variation in candidate genes on imaging correlates in the general population and offers the opportunity to study brains not influenced by chronic disease and medication. Previous studies showed that neuroimaging correlates of common genetic variants are likely to be similar in typical and psychiatric populations (Hibar et al., 2015b). As such studies of healthy individuals may also be informative regarding the biological mechanisms leading to the diseases of interest, they were also included in this review.

Methods

Search terms

Pubmed was searched for research articles describing imaging genetics studies (April, 14th, 2015; http://www.ncbi.nlm.nih.gov/pubmed). Only studies using magnetic resonance imaging (MRI) were reviewed, specifically structural MRI (sMRI), functional MRI (fMRI), resting-state functional MRI (rs-fMRI), and diffusion tensor imaging (DTI). A general search term was created and was extended by adding the disorder (for ADHD and ASD) or syndrome name and gene (for ID) of interest. The following search term shows an example for ADHD (for [Title/Abstract]): ((((ADHD OR Attention-Deficit Hyperactivity Disorder) AND (gene* OR genetic* OR imaging genetic OR imaging genetics OR genotype OR polymorphism OR SNP OR single nucleotide polymorphism OR meta-analysis OR genome wide association OR GWA OR GWAS)) AND (structural magnetic resonance imaging OR volume OR sMRI OR voxel-based morphometry OR brain morphometry OR brain volumetry OR VBM OR functional magnetic resonance imaging OR fMRI OR diffusion tensor imaging OR diffusion imaging OR connectivity OR tractography OR DTI OR resting-state functional magnetic resonance imaging OR voxel-wise analysis OR rsfMRI)) NOT "review"[Publication Type]). For ID syndromes, the search term did not include (gene* OR genetic* OR imaging genetic OR imaging genetics OR genotype OR polymorphism OR SNP OR single nucleotide polymorphism OR meta-analysis OR genome wide association OR GWA OR GWAS), as the genes of interest were added specifically. Titles and abstracts of the retrieved records were evaluated for relevant publications. Case-reports and reports describing less than 10 cases were excluded to prevent bias, and review articles, medical hypotheses, non-English articles, and studies on animal models were not considered (for a graphical summary of the selection procedure, please see **Figure 1**).

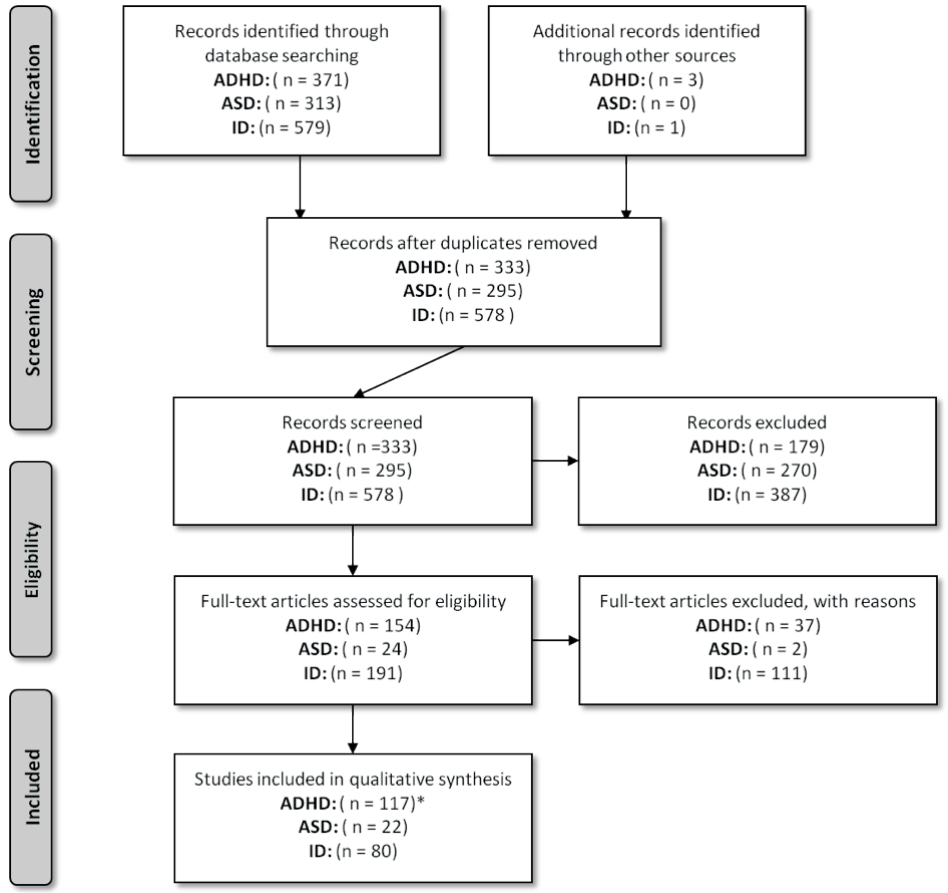

Figure 1. Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA) flowchart of the literature search and study selection for qualitative analysis. Note: see http://www.prismastatement.org/ for more information on this reporting system. ADHD = Attention-Deficit/Hyperactivity Disorder, ASD = Autism Spectrum Disorder, ID = Intellectual Disability. Records excluded for ID contain unrelated records identified by screening as well as records describing non-ID samples. The number of studies for ADHD and ASD * The number of studies for ADHD candidate genes also include the records for *SLC6A4* (5-HTTLPR), which is also a candidate gene for ASD.

Candidate gene selection for ADHD, ASD, and ID studies

Taking into account the differences in the genetic architecture of the three neurodevelopmental disorders of interest, we defined selection criteria for the genes to be included in this review as similar as possible. The restriction to studies with 10 or more cases and single genetic variants/single-gene mutations largely defined our search strategy, which resulted in a focus on common genetic variants for ADHD and ASDs (minor allele frequency ≥ 1%); for ID disorders, this lead to the selection of relatively common forms of the disorder. For ADHD and ASDs, we selected the most promising genes containing common variants

associated with the disorder based on meta-analyses, successful replication studies, and/or significant findings from hypothesis-free (genome-wide) studies.

For ADHD, we included all genes and genetic variants mentioned in Table 1 of the meta-analytic study by Gizer and coworkers (2009) that had reached a significant result at $P \leq 0.05$ for association with ADHD. In addition to this, we also included genes with reported and replicated evidence for association with ADHD from more recent studies. These included two meta-analytic studies (Pan et al., 2015; Wu et al., 2012), a research article (Ribases et al., 2011), and the more recently observed replicated candidate genes *NOS1* and *SLC9A9* (Stergiakouli et al., 2012; Weber et al., 2015) (total number of candidate genes = 10; **Table 1**). A recent overview of these ADHD candidate genes has been published by Hawi and colleagues (2015).

For the ASD genes, we based our selection on the review of the most consistently replicated genes harboring common variants associated with autism by Persico and Napolioni (2013). Additionally, the *CDH9/CDH10* locus was included, because it has shown genome-wide significant association with ASD (Prandini et al., 2012; Wang et al., 2009). Selection of the candidate polymorphisms in the selected genes was based on recent research articles, as meta-analyses were only available for the *OXTR* and *RELN* gene (total number of candidate genes = 11; **Table 2**).

For the ID, the restrictions to relatively common forms of the disorder resulting from single gene mutations (as opposed to structural genetic variants involving several to many genes) as well as our aim to study potential brain mechanisms contributing to comorbidity among the three disorders lead to the inclusion of the following 5 syndromes: fragile X syndrome (*FMR1*), tuberous sclerosis (*TSC1* and *TSC2*), neurofibromatosis type 1 (*NF1*), Rett syndrome (*MECP2*), and Timothy syndrome (*CACNA1C*) (**Table 3**). For our selection, we used Table 1 from Vorstman and Ophoff (2013), describing genetic anomalies associated with ID. We included all disorders with known genetic cause including a single gene (*FMR1, TSC1* and *TSC2, NF1, and CACNA1C*). Patients with these disorders also show a high rate of ASD and/or ADHD phenotypes (Vorstman and Ophoff, 2013). Additionally, we included the Rett syndrome (*MECP2*), because of its known ASD- and ADHD-related features (Armstrong, 2005; Rose et al., 2016; Suter et al., 2014).

Table 1: Genes containing common variants most consistently implicated in ADHD, based on (Gizer et al., 2009) and more recent (meta-)analyses.

Gene	Protein	Associated variant/ polymorphism	Risk allele	Location/chr position	References for reports of association with ADHD
DRD2/ ANNK1	Dopamine receptor D2/ Ankyrin repeat and kinase domain containing 1	Taq1A (rs1800497)	T allele = A1-allele	Exon 8/ 3′ flanking/11q23	(Comings et al., 1991)[a]; (Pan et al., 2015)[b]
*DRD4**	Dopamine receptor D4	**48 bp VNTR**	7 repeat (5 repeat in Asians)	Exon 3/11p15	(LaHoste et al., 1996)[a]; (Gizer et al., 2009)[b]; (Wu et al., 2012)[b]
		rs1800955	T allele	Promoter/11p15	(Barr et al., 2001)[a]; (Yang et al., 2008)[d]; (Gizer et al., 2009)[b]
DRD5	Dopamine receptor D5	**148 bp dinucleotide repeats**	148 bp allele	5′ flanking/4p16	(Daly et al., 1999)[a]; (Gizer et al., 2009)[b]; (Wu et al., 2012)[b]
HTR1B	Serotonin receptor 1B, G protein-coupled	**rs6296**	G allele	Exon 1/6q14	(Hawi et al., 2002)[a]; (Gizer et al., 2009)[b]
LPHN3	Latrophilin 3	rs6551665 rs6858066	G allele G allele	4q13	(Arcos-Burgos et al., 2010)[a]; (Hwang et al., 2015)[d]; (Ribases et al., 2011)[d]; (Labbe et al., 2012)[a]
*NOS1**	Nitric oxide synthase 1	180-210 bp CA repeat	Short allele	Exon 1/12q24	(Reif et al., 2009)[a]; (Franke et al., 2009)[c]; (Weber et al., 2015)[b]
*SLC6A3/ DAT1**	Solute Carrier Family 6 (Neurotransmitter Transporter), Member 3; Dopamine transporter 1	**40 bp VNTR**	10 repeat	3′ UTR/5p15	(Cook et al., 1995)[a]; (Gizer et al., 2009)[b]
		rs27072	G allele	3′ UTR/5p15	(Galili-Weisstub and Segman, 2003)[a]; (Gizer et al., 2009)[b]
		30 bp VNTR	6 repeat	Intron 8/5p15	(Brookes et al., 2006)[a]; (Gizer et al., 2009)[b]
*SLC6A4/ 5HTT**	Solute carrier family 6 (neurotransmitter transporter), member 4; serotonin transporter	**5-HTTLPR**	Long allele	Promoter/17q11	(Manor et al., 2001)[a]; (Gizer et al., 2009)[b]; (Landaas et al., 2010)[b]
SLC9A9/ NHE9	Solute Carrier Family 9, Subfamily A, Member 9	rs9810857	T allele	Region 3p14-q21	(de Silva et al., 2003)[a]; (Stergiakouli et al., 2012)[c]; (Mick et al., 2010)[c]
SNAP25	Synaptosomal-associated protein, 25kDa	**rs3746544**	T allele	3′ UTR/20p12	(Brophy et al., 2002)[a]; (Gizer et al., 2009)[b]

Bold text indicates significant result at $P < 0.05$ in Gizer et al., 2009. [a]Association first reported by. [b]Meta-analysis article. [c]GWAS finding. [d]Association in large sample or validation using animal model. *Gene with at least one case-control imaging genetics study; ADHD = Attention deficit/hyperactivity disorder, bp = base pair, chr = chromosome, CNV = copy number variation, UTR = untranslated region, VNTR = variable number tandem repeat; in grey no imaging genetics studies found.

Table 2: Genes containing common variants most convincingly implicated in ASDs, adapted from Persico and Napolioni (2013). We added *CDH9, CDH10,* and *MSNP1AS*, because the locus harbouring these genes has shown genome-wide significant association with ASDs in GWAS (Prandini et al., 2012; Wang et al., 2009). Selection of candidate polymorphisms and risk alleles for ASD was based on recent research articles.

Gene	Protein	Associated variant/ polymorphism	Risk allele	Location/chr position	References for association with ASD
CDH9	Cadherin 9	rs4307059	C allele	Intergenic/5p14	(Wang et al., 2009)[a,c]; (Prandini et al., 2012)[d]
CDH10	Cadherin 10	rs4307059	C allele	Intergenic/5p14	(Wang et al., 2009)[a,c]; (Prandini et al., 2012)[d]
MSNP1AS	Moesin pseudogene 1, antisense	rs4307059	C allele	Intergenic/5p14	(Wang et al., 2009)[a,c]; (Prandini et al., 2012)[d]
*CNTNAP2**	Contactin associated protein-like 2	rs7794745	T allele	Intron 2/7q35	(Arking et al., 2008)[a]; (Li et al., 2010)[d]
		rs2710102	C allele	Exon 8/7q35	(Stein et al., 2011)
EN2	Engrailed homeobox 2	rs1861972	G allele	Intron/7q36	(Gharani et al., 2004)[a]; (Benayed et al., 2005)[d]
		rs1861973	T allele	Intron/7q36	(Gharani et al., 2004)[a]; (Benayed et al., 2005)[d]
GABRB3	Gamma-aminobutyric acid (GABA) A receptor, beta 3	rs7171512	G allele	Intron/15q12	(Warrier et al., 2013)[a]
		rs7180158 (AS)	G allele	Intron/15q12	(Warrier et al., 2013)[a]
		rs7165604 (AS)	T allele	Intron/15q12	(Warrier et al., 2013)[a]
		rs12593579 (AS)	C allele	Intron/15q12	(Warrier et al., 2013)[a]
		rs9806546 (EQ)	G allele	Intron/15q12	(Warrier et al., 2013)[a]
		rs11636966 (EQ)	T allele	Intron/15q12	(Warrier et al., 2013)[a]
ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	rs12603582	T allele	Intron 11/17q21.32	(Napolioni et al., 2011)[a]; (Schuch et al., 2014)[d]
		rs15908	A allele	Exon 9/17q21.32	(Schuch et al., 2014)[a]
*MET**	Met proto-oncogene (hepatocyte growth factor receptor)	rs1858830	C allele	Promoter/7q31	(Campbell et al., 2006)[a]; (Sousa et al., 2009)[d]; (Thanseem et al., 2010)[d]; (Zhou et al., 2011)[d]

Table 2: Continued.

Gene	Protein	Associated variant/ polymorphism	Risk allele	Location/chr position	References for association with ASD
OXTR	Oxytocin receptor	rs7632287	A allele	3′ flanking/3p25	(Tansey et al., 2010)[a]; (LoParo and Waldman, 2014)[b]; (Campbell et al., 2011)[d]
		rs237887	A allele	Intron3/3p25	(Liu et al., 2010)[a]; (LoParo and Waldman, 2014)[b]
		rs2268491	T allele	Intron3/3p25	(Liu et al., 2010)[a]; (LoParo and Waldman, 2014)[b]
		rs2254298	A allele	Intron3/3p25	(Wu et al., 2005)[a]; (LoParo and Waldman, 2014)[b]; (Liu et al., 2010)[d]; (Nyffeler et al., 2014)[d]
		rs2268493	C allele	Intron3/3p25	(Yrigollen et al., 2008)[a]; (Campbell et al., 2011)[d]; (Di Napoli et al., 2014)[d]
		rs53576	A allele	Intron3/3p25	(Wu et al., 2005)[a]; (Nyffeler et al., 2014)[d]
		rs2268494	T allele	Intron3/3p25	(Lerer et al., 2008)[a]
RELN	Reelin	rs362691	Population specific?	Exon 22/7q22	(Wang et al., 2014b)[b]
		rs362780	G allele	Intron 41/7q22	(Holt et al., 2010) [a]
		rs736707	Population specific?	Intron 59/7q22	(Sharma et al., 2013)[a]
		rs2073559	T allele	Intron 11/7q22	(Ashley-Koch et al., 2007) [a]
*SLC6A4/ 5HTT**	Serotonin transporter	5-HTTLPR	Long allele	Promoter/17q11.2	(Nyffeler et al., 2014)[d]; (Gadow et al., 2013)

[a]Association first reported by. [b]Meta-analysis article. [c]GWAS finding. [d]Association in large sample or validation using animal model. *Gene with at least one case-control imaging genetics study; ASD = Autism spectrum disorder, AS = Asperger's syndrome, chr = chromosome, EQ = empathy quotient); in grey no imaging genetics studies found.

Table 3: Genes causing prevalent and well-studied single-gene ID disorders with behavioral and cognitive overlap with ADHD and/or ASD.

Gene	Protein	Chr position	Associated ID disorder	Reported rate of ASD-related phenotype	Reported rate of ADHD-related phenotype
FMR1	Fragile X mental retardation protein	Xq27	Fragile X syndrome	30% (Hagerman et al., 2009)	59% (Sullivan et al., 2006)
NF1	Neurofibromin	17q11	Neurofibromatosis type 1	40% (Walsh et al., 2013)	38% (Hyman et al., 2005)
TSC1 *TSC2*	Hamartin Tuberin	9q34 16p13	Tuberous sclerosis complex	50% (Prather and de Vries, 2004)	30-60% (D'Agati et al., 2009)
MECP2	Methyl-CpG-binding protein 2	Xq28	Rett syndrome	42-58% (Wulffaert et al., 2009)	unknown
CACNA1C	Voltage-dependent L-type calcium channel subunit alpha-1C	12p13	Timothy syndrome	60% (Splawski et al., 2004)	unknown

Phenotypic overlap as adapted from (Vorstman and Ophoff, 2013); ID= intellectual disability; ASD= Autism spectrum disorder; ADHD= Attention deficit/hyperactivity disorder; Chr= chromosome; in grey no imaging genetics studies found.

Results

Imaging genetics of ADHD candidate genes

A total of 76 records were retrieved for the ADHD search term, and a total of 16 research articles describing case-control studies were eligible for review according to our criteria. To those, we added three more recent papers from our own group ((Onnink et al., 2016; Sokolova et al., 2015; van der Meer et al., 2015); **Figure 1**). Most of the studies investigated a single gene (all in Caucasians), and three studies investigated multiple genes (2 in Caucasians, 1 in Asians). In addition, we obtained 295 records for the ADHD candidate gene studies in healthy population samples, of which 98 were eligible (**Figure 1**). Of those, 73 studies investigated a single gene (68 in Caucasians, 5 in Asians), and 25 studies tested more than one gene (1 Asian). The ADHD case-control samples consisted of both childhood/adolescent and adult samples, whereas the studies in the healthy population were largely restricted to samples of (young) adults. Single-gene findings of ADHD case-control studies and studies in the healthy population of both Caucasian and Asian ethnicities can be found in **Table 4,** multi-locus studies are shown in **Table 6**. Most of the genes investigated in brain imaging genetics studies in ADHD are from the dopaminergic and serotonergic neurotransmitter systems (*SLC6A3/DAT1*, *DRD2*, *DRD4*, *SLC6A4/5-HTT/SERT*). *SNAP25, DRD5, HTR1B,* and *LPHN3* had also been selected for this study, but for these genes no imaging genetics studies using MRI were found with our search terms.

The **dopamine transporter gene** *DAT1* (official name *SLC6A3*) codes for a solute carrier protein, responsible for the reuptake of dopamine from the synaptic cleft into the

presynaptic neuron, representing a primary mechanism of dopamine regulation in the striatum (Ciliax et al., 1999). The most widely studied polymorphism in *SLC6A3/DAT1* is a variable number of tandem repeat (VNTR) sequence in the 3' untranslated region (3'UTR) that is 40 base pairs (bp) in length. Most common alleles are those with 9 and 10 repeats. Additionally, a 30 bp VNTR in intron 8 of the gene (most common alleles with 5 and 6 repeats), is sometimes studied together with the 3'UTR VNTR as a haplotype. The 10R/10R genotype of the 3'UTR VNTR and the 10-6 haplotype of the two VNTRs are thought to be risk factors for ADHD in children (Asherson et al., 2007; Brookes et al., 2006; Faraone et al., 2005). In contrast, the 9R/9R genotype and the 9-6 haplotype are associated with persistent ADHD (Franke et al., 2010). The sMRI and fMRI studies for *SLC6A3/DAT1*, the latter investigating several cognitive domains known to be impaired in ADHD, i.e. reward processing, working memory, and response inhibition, are summarized in **Table 4** and **6**. The main focus of the studies for this gene has clearly been on the striatum, which shows highest gene expression.

The two sMRI case-control studies were performed in children, and both reported a smaller volume of the caudate nucleus in homozygotes for the 10R allele as compared to children with the 9R/10R genotype (Durston et al., 2005; Shook et al., 2011). A third study, including a large sample of children and adults with and without ADHD, showed that only in the adult ADHD case-control cohort, carriers of the *DAT1* adult ADHD risk haplotype 9-6 had a 5.9% larger striatum volume relative to participants not carrying this haplotype. The effect was depended on diagnostic status, since the risk haplotype affected striatal volume only in patients with ADHD (Onnink et al., 2016).

Two fMRI studies in case-control design investigated the *SLC6A3/DAT1* haplotype using reward paradigms. Independent of the genotype, a recent meta-analysis has shown that in reward-processing paradigms, most studies report lower activation of the ventral striatum in patients with ADHD in anticipation of reward than controls (Plichta and Scheres, 2014). Consistent with this, a study in adolescents (including only males) found the activation of the caudate nucleus to be reduced in the ADHD group as the number of 10-6-haplotype copies increased (Paloyelis et al., 2012). The other study, in adult ADHD cases and controls (in whom the 9-6 allele is the ADHD risk allele), found no effect of *DAT1* haplotype on striatal activity (Hoogman et al., 2013). Studies in healthy adult individuals point in different directions. One found higher activation during reward anticipation in 9R-carriers (Dreher et al., 2009). Another also found increased striatal activation in 9R-carriers in a rewarded task-switching task, especially in high reward conditions (Aarts et al., 2010). A third study in healthy adults suggested that a link between reward sensitivity and striatal activation during reward anticipation is only present in 10R/10R individuals, and is lost in 9R-carriers (Hahn et al., 2011). In studies of response inhibition in children/adolescents, the 10R/10R genotype was found linked to lower (Durston et al., 2008) but also higher (Bedard et al., 2010) striatal activation. Methylphenidate was able to increase activity in the caudate nucleus (as well as a thalamocortical network and inferior frontal gyrus) during successful inhibition in

healthy adult male 9R-carriers, but decreased activity in 10R/10R individuals (Kasparbauer et al., 2015). A working memory task in healthy adults elicited more activation in fronto-striatal-parietal regions in 9R/10R individuals under high memory load (Stollstorff et al., 2010). Additionally, a resting-state fMRI study in healthy adults showed stronger connectivity between midbrain (mainly striatal) and prefrontal regions in 9R/10R heterozygotes compared with 10R/10R homozygotes (Gordon et al., 2015).

Beyond striatum, *SLC6A3/DAT1* genotype effects have also been observed in fMRI studies of cortical regions, especially (pre)frontal, medial (pre-SMA, dorsal ACC), and (temporo) parietal regions (Bedard et al., 2010; Braet et al., 2011) (**Table 4 and 6**). As expression of DAT is limited outside of striatum and cerebellum, these effects are likely due to direct or indirect connections between the regions of gene expression and the rest of the brain. This is in line with the fact that no effect of *SLC6A3/DAT1* genotype on cortical development has been observed in a longitudinal study (Shaw et al., 2007). Of particular interest might be studies showing effects of *SLC6A3/DAT1* genotype on amygdala reactivity upon exposure to threatening faces (Bergman et al., 2014) as well as on cerebellar activation during response inhibition (Durston et al., 2008). These regions are currently understudied in ADHD. A first study using DTI did not suggest a strong effect of *SLC6A3/DAT1* genotype on structural brain connectivity (Hong et al., 2015) (**Table 4**).

In summary, although *SLC6A3/DAT1* is one of the best-studied genes in imaging genetics literature covered in this review, existing studies do not yet clarify sufficiently the role of ADHD-linked genetic variation in brain activity and connectivity related to symptoms/cognitive deficits or their structural brain correlates. A complicating matter for this gene is the switch in ADHD risk allele from childhood to adulthood. Furthermore, interactions between genotype and diagnosis are observed in some studies, which suggest that studying effects of *SLC6A3/DAT1* in healthy individuals will not suffice to fully understand the brain mechanisms linking this gene to ADHD.

The **dopamine D2 receptor** gene (*DRD2*) codes for a G protein-coupled receptor, which inhibits adenylate cyclase (Andersen et al., 1990). Consistent with its broad expression in the brain being highest in striatum, *DRD2* plays a key role in regulating mesolimbic reward processing pathways (Usiello et al., 2000) and is also implicated in other cognitive domains, such as cognitive flexibility and learning (Puig et al., 2014). The gene has been implicated in many different psychiatric disorders, including schizophrenia and substance use disorders (Patriquin et al., 2015; Schizophrenia Working Group of the Psychiatric Genomics, 2014) and is the target of several antipsychotics (Moore et al., 2014). The risk factor for ADHD is the most frequently investigated common genetic variant of *DRD2* rs1800497 (also known as Taq1A restriction fragment length polymorphism). This SNP actually lies downstream of *DRD2* in an exon of a neighboring gene, *ANKK1* (Neville et al., 2004). It affects dopamine D2 receptor expression and striatal dopamine metabolism, with the A1-allele (the ADHD risk allele) reducing the number of *DRD2* receptors (Laakso et al., 2005). No studies in ADHD

case-control design are yet available for *DRD2*. The risk SNP has, however, been investigated in healthy individuals using structural and functional MRI covering the cognitive domains of reward processing, task-switching and reversal learning, working memory, emotion recognition, and language (**Table 4** and **6**).

Structural MRI showed that the SNP affects the volume of midbrain structures, with A1-allele carriers having smaller volumes of substantia nigra (Cerasa et al., 2009), cerebellum (Wiener et al., 2014), and ACC (in interaction with *BDNF*; (Montag et al., 2010)).

Functional MRI during reversal learning tasks revealed that A1-allele carriers showed reduced response of the rostral cingulate to negative feedback and had a reduced recruitment of the right ventral striatum and right lateral occipital frontal cortex (OFC) during reversals (Jocham et al., 2009). Pharmacological fMRI in a reversal learning task showed that cabergoline (D2 receptor agonist) administration induced an allele-specific response, where A1-allele carriers showed increased neural reward responses in medial OFC, cingulate cortex, and striatum (consistent with increased D2-mediated dopamine signaling); this was coupled, however, to worse task performance and lower fronto-striatal functional connectivity (Cohen et al., 2007). The reward-related paradigms showed that A1-allele carriers exhibited increased anterior insula (Richter et al., 2013) and increased nucleus accumbens activation, the latter observed only in a three-way interaction analysis looking for differences between a placebo and bromocriptine (D2 receptor agonist) administration condition (Kirsch et al., 2006). Two multi-locus studies including the *DRD2* Taq1A variant suggested higher activation during reward anticipation, but blunted activity during reward receipt with increasing number of risk factors (**Table 6**).

In summary, the effects of the ADHD risk factor in *DRD2* in fMRI appear to be relatively consistent across most of the studies currently available, with stronger brain activity in parts of the wider reward processing and memory/learning circuits. It seems that this stronger activity is linked to worse functional connectivity and/or performance, thus potentially reflecting compensatory processes. Currently, no data from patients with ADHD are available.

The **dopamine D4 receptor** (encoded by the *DRD4* gene) is another G protein-coupled receptor and belongs to the dopamine D2-like receptor family (Oldenhof et al., 1998). The most widely studied *DRD4* polymorphism in ADHD has been the 48 bp VNTR in exon 3, with the 2-, 4-, and 7-repeat alleles being the most common alleles. Allele frequencies vary significantly across ethnic groups (Chang et al., 1996; Van Tol et al., 1992), and the ADHD risk allele in the Caucasian population (7R) seems to be a different one from that in Asians (Nikolaidis and Gray, 2010; Wang et al., 2004).

Structural MRI suggested that patients with ADHD carrying the 7R-allele have smaller volumes of the superior frontal and cerebellar cortex (Monuteaux et al., 2008), while no differences were found in another study (Castellanos et al., 1998) (**Table 4**). Interestingly, carriership of the *DRD4* 7R-allele seemed to affect cortical development in a longitudinal

study, with 7R-carriers showing thinner prefrontal and parietal cortex and ADHD patients with this allele having a distinct trajectory of cortical development characterized by normalization of parietal cortical regions (Shaw et al., 2007) (**Table 6**). Structural connectivity was investigated in two studies in Asians using DTI, and while one did not find effects for 4R homozygotes (Hong et al., 2015), a very large recent study reported widespread increases in mean diffusivity in 5R-carriers (Takeuchi et al., 2015) (**Table 4**).

With the role of the D4 dopamine receptor in cognition not sufficiently characterized yet, and *DRD4* being expressed in large parts of the cortex (predominantly in frontal lobe regions, such as the OFC and ACC (Floresco and Tse, 2007; Noain et al., 2006)), fMRI studies have investigated the *DRD4* gene in healthy Caucasians covering different cognitive domains, i.e. emotion processing, response inhibition, reward, stimulus-response incompatibility, and time discrimination tasks, as summarized in **Table 4**. Depending on the type of paradigm used in the fMRI studies, *DRD4* genotype was found to modulate brain activity in prefrontal and temporal, but also in striatal and cerebellar brain regions in the healthy adults (**Table 4**).

Thus, though existing evidence does not support firm conclusions, *DRD4* may mark a particular developmental trajectory in cortical brain structure related to adult outcome of ADHD, and plays a role in structural connectivity. With only one fMRI study per cognitive domain published to date, no clear picture of *DRD4* action on brain activity emerges, but those studies do clearly indicate that *DRD4* (like *DAT1*) influences brain activity beyond its regions of expression, possibly due to its effects on white matter connectivity (Takeuchi et al., 2015).

The **serotonin transporter** gene (*SLC6A4*, *5HTT, SERT*) codes for a solute carrier protein responsible for the reuptake of serotonin from the synaptic cleft back into the presynaptic neuron, which is the primary mechanism for regulation of serotonergic activity in the brain (Lesch et al., 1996). A functional polymorphism in the promoter region of the gene (referred to as 5HTTLPR) is a 44-bp insertion/deletion yielding short (S) and long (L) alleles. The long variant is associated with more rapid serotonin reuptake, resulting in lower levels of active serotonin (Lesch et al., 1996). However, allele frequencies vary across different ethnic groups (Haberstick et al., 2015). A SNP in the long allele, rs25531, can modify the activity of this allele (Lesch et al., 1996). *SLC6A4/5HTT* has been implicated in emotion regulation as well as (emotional) memory and learning processes (Araragi and Lesch, 2013; Barzman et al., 2015; Meneses and Liy-Salmeron, 2012). Expression of the transporter is observed in regions implicated in attention, memory, and motor activities, such as the amygdala, hippocampus, thalamus, putamen, and ACC (Frankle et al., 2004; Oquendo et al., 2007).

Only one recent imaging genetics study in patients with ADHD has been performed for the 5HTTLPR, showing that stress exposure, which is associated with increased ADHD severity in S-allele carriers, was associated with reduced cortical gray matter volume in precentral gyrus, middle and superior frontal gyri, frontal pole, and cingulate gyrus in these individuals. Interestingly, this paper showed that only some of these regions, the frontal

pole and the ACC, actually mediated the effects of the gene-environment interaction on ADHD severity. In sMRI studies in healthy individuals, the 5HTTLPR has been associated with volume of the ACC and amygdala as well as hippocampus, though the direction of effect seemed to differ with gender and/or in interaction with environmental factors (**Table IV**). Few studies have looked at effects of the 5HTTLPR on structural connectivity (**Table 4**). A large study observed reduced connectivity of amygdala with PFC in S-allele carriers (Long et al., 2013), while another reported increased hippocampus-putamen connectivity for this genotype group (Favaro et al., 2014).

Brain activation patterns in task-based fMRI have been studied extensively for the 5HTTLPR following hallmark studies by the Weinberger lab (Hariri et al., 2005; Hariri et al., 2002). They were the first to report increased activation of the amygdala in S-allele carriers in response to negative-emotional faces. Since then, increased amygdala activation has been observed in S-allele carriers in many tasks activating the amygdala (**Table 4** and **6**). In 2013, 34 studies investigating effects of the 5HTTLPR on amygdala activation were meta-analyzed, confirming the increased activation in S-allele carriers (although only borderline significant) (Murphy et al., 2013). However, this meta-analysis also showed strong heterogeneity between studies and a potential publication bias (towards studies reporting significant associations). Linked to the increased activation seems to be a reduced functional connectivity of the amygdala, as first observed by Pezawas and colleagues (2005) and subsequently also seen in additional studies (**Table 4**). Not only the amygdala, but also other cortical and subcortical brain regions (forming the 'threat circuit') seem to be influenced by 5HTTLPR genotype. A recent, replicated fMRI study, for example, also showed stronger activity in dorsomedial prefrontal cortex (dmPFC), insula, thalamus, and regions of the midbrain, in reaction to threat in S-allele carriers (Klumpers et al., 2014); interestingly, also in this study (like in the one by van der Meer and coworkers (2015)) only some of the activated regions actually mediated the genotype effects on psychophysiological responsivity to pending threats (in this case the dmPFC activation, **Table 4**).

Increasing evidence suggests that S-allele carriers are hypervigilant to environmental stimuli (Homberg and Lesch, 2011). Potential sustained effects of environmental factors have not sufficiently been addressed in imaging genetics studies published to date. Several studies have taken stressful life events into account, and these studies suggested effects on both brain volume and activation. Only one study to date has directly looked at methylation of the promoter of the *SLC6A4/5HTT* gene, and found correlations with the volume of several regions in the 'threat circuit' of the brain, though these appeared genotype-independent (Dannlowski et al., 2014). Also a combined PET, sMRI plus fMRI study indicated that 5HTTLPR genotype did not influence current (midbrain) serotonin transporter availability (Kobiella et al., 2011), suggesting that other factors (like environmental ones) might overrule this effect. Taking into account epigenetic effects on the *SLC6A4/5HTT* gene might thus help

explain the strong heterogeneity observed in the meta-analysis of amygdala reactivity studies (Murphy et al., 2013).

In summary, functional genetic variation in the *SLC6A4/5HTT* gene is clearly linked to emotion regulation through effects on brain activation in the amygdala and the wider 'threat circuit', with those carrying the risk factor for emotional dysregulation showing increased activation in tasks related to emotion processing and learning. Those experiments link reduced availability of the transporter (at some point in development) - and thus increased serotonin signaling capacity - to increased brain activation. This increased activation seems to be linked to functional dysconnectivity, however. Whether brain volume and structural integrity are influenced by the 5HTTLPR, remains to be clarified. Importantly, genotype effects are likely to be sensitive to environmental factors.

The **nitric oxide synthase 1** (encoded by the *NOS1* gene) is an enzyme which synthesizes nitric oxide from L-arginine. Nitric oxide is a reactive free radical, which acts as a biological mediator in several processes, including dopaminergic and serotonergic neurotransmission (Kiss and Vizi, 2001). The *NOS1* gene has a complex structure, including 12 alternative untranslated first exons (exon 1a-1l). In exon 1f, a functional VNTR that affects gene expression has been linked to hyperactive and impulsive behavior in humans (Reif et al., 2009; Weber et al., 2015), with the short allele being the risk factor for ADHD. In addition, a recent *Nos1* knock-out mouse model showed dysregulation of rhythmic activities mimicking ADHD-like behaviors (Gao and Heldt, 2015).

So far, only one case-control study investigated the effect of the VNTR polymorphism on the brain, in his case on reward-related ventral striatal activity (Hoogman et al., 2011) (**Table 4**). The study revealed that homozygous carriers of the short allele of *NOS1* demonstrated higher ventral striatal activity than carriers of the other *NOS1* VNTR genotypes (Hoogman et al., 2011). This effect was comparable for both patients and healthy individuals. Similar effects of the genotype were also observed for behavioral impulsivity, with those carrying the ADHD risk factor acting more impulsive than other participants.

Table 4: Imaging genetics studies in ADHD case-control samples and ADHD candidate genes studies in the healthy population (for selection of candidate genes see **Table 1**).

Gene	Variant	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
DRD2	DRD2/ ANKK1-Taq1a (rs1800497, T allele = A1 allele)	sMRI (VBM)	Global GM volume	A1- carriers vs. A2/ A2-carriers	70 HC (30.7)	A1- carriers: ↓ part of midbrain, encompassing substantia nigra bilaterally	(Cerasa et al., 2009)
		sMRI (VBM)	GM and WM volume	A1- carriers vs. A2/ A2-carriers	25 HC (25)	A1-carriers: ↓ Volume in cerebellar cluster	(Wiener et al., 2014)
		fMRI	Temporal or color discrimination task			A1-carriers: ↑ Activation in striatum and right dorsolateral PFC	
			Reward anticipation paradigm	A1- carriers vs. A2/ A2-carriers	24 HC (25.7)	↑ Nucleus accumbens activation in three-way interaction analysis from placebo to bromocriptine (D2 receptor agonist); ↑ performance under bromocriptine in A1- carriers.	(Kirsch et al., 2006)
			Striatal activation in response to receiving palatable food (2 fMRI paradigms)	A1- carriers vs. A2/ A2-carriers	fMRI 1: 43 HC (20.4) † fMRI 2: 33 HC (15.7) †	↑ Negative relation between striatal response to food receipt and BMI. A1-non-carriers : striatal activation in response to food intake was positively related to weight gain (negatively related to weight gain for A1- carriers).	(Stice et al., 2008)
			Emotional face task	A1/A1-carriers vs. A1/A2-carriers vs. A2/A2-carriers	45 HC (23.2)†§	TaqIA genotype modifies activations in putamen, ACC, and amygdala in response to negative facial stimuli (higher signal intensity in homozygous groups (A1/A1 + A2/A2) than in heterozygous group (A1/A2)).	(Lee et al., 2011)
			Flanker task with a motivation manipulation	A1- carriers vs. A2/ A2-carriers	32 HC (22.9)	A1- carriers: ↓ Interference effects to reward alone (as compared to reward + punishment) and ↑ anterior insula activation	(Richter et al., 2013)
			Task-switching paradigm	A1-non-carriers vs. A1- carriers	48 HC (22)	A1 non-carriers: ↑ Task-switching costs, ↑ prefrontal switching activity in inferior frontal junction area, and ↑ functional connectivity in dorsal frontostriatal circuits	(Stelzel et al., 2010)

Table 4: Continued

Gene	Variant	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
			Feedback-based reversal learning task	A1- carriers vs. A2/ A2-carriers	22 HC (age range 20–31)	A1- carriers in placebo condition: ↓ neural responses to positive feedback; cabergoline: ↑neural reward responses in medial OFC, cingulate cortex, and striatum, but ↓task performance and fronto-striatal functional connectivity	(Cohen et al., 2007)
			Probabilistic reversal learning task	A1- carriers vs. A2/ A2-carriers	28 HC (26.1)‡	A1- carriers: no graded increase in RCZ activity to preceding negative feedback; ↓ recruitment of right VS and right lateral OFC during reversals.	(Jocham et al., 2009)
			"Wug" test (knowledge of grammar, opposed to vocabulary)	A2/A2-carriers vs. A1- carriers	22 HC (22)	A2/A2-carriers: ↑ At concatenative (but not analogical) grammar learning; ↑ striatal responses	(Wong et al., 2013b)
DRD4	exon 3 VNTR #	sMRI	Superior frontal, middle frontal, anterior cingulate, and cerebellum cortices volumes	ADHD 7R-carriers vs. non 7R-carriers	24 ADHD (38.1) 19 ADHD+BPD (35.8) 20 HC (33.2)	7R- carriers: ↓ volumes of superior frontal cortex and cerebellum cortex compared to non-carriers. No effects in ADHD+BPD or HC.	(Monuteaux et al., 2008)
			TBV, PFC, cerebellum, CN and pallidum volume	7R-carriers vs. non-7R-carriers	41 ADHD (9.7) 56 HC (17.6)	No volumetric differences between 7R-carriers and non-7R-carriers. No group x genotype interactions.	(Castellanos et al., 1998) ‡
	exon 3 VNTR	DTI	WM integrity	5R- carriers vs. non 5R-carriers	765 HC (20.7) §	5R-carriers : ↑ MD in widespread GM and WM areas of cerebral cortex, and subcortical areas	(Takeuchi et al., 2015)
		fMRI	Activity related to N-back paradigm			5R-carriers : ↓ Task-induced deactivation in precuneus areas in both attention-demanding working memory task and sensorimotor task; similar patterns were observed in posterior cingulate cortex and areas around midbrain and hippocampus.	

Table 4: Continued

Gene	Variant	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
		fMRI	MID task	7R-carriers vs. non 7R-carriers	78 HC (16.3)	*DRD4* status moderated relation between Behavioral Inhibition (BI) and activation in CN. 7R-carriers: ↑ striatal response to incentive cues. DRD4 genotype influenced relations among neural response to incentives, early childhood BI and anxiety.	(Perez-Edgar et al., 2014)
			Emotional rating task	4R/7R-carriers vs. 4R/4R-carriers	26 HC (23.3)	4R/7R-carriers: ↑ activity in response to unpleasant images compared to neutral images in right temporal lobe.	(Gehricke et al., 2015)
			Go/No-go task	7R-carriers vs. non 7R-carriers	62 HC (18)	7R-carriers "No-Go" trials:, ↓ activation in right anterior PFC/ IFG, left premotor cortex, and right occipital/ cerebellar areas (7-repeat status accounted for ca. 5-6% of variance in BOLD response during "No-Go" trials).	(Mulligan et al., 2014)
			Combined stimulus-response Incompatibility Task (IC) and Time Discrimination Task (TT)	7R-non-carriers vs. 7R-carriers	26 HC (11.4)	7R-non-carriers: ↑activation of left middle and IFG in IC and ↑cerebellar activation in TT; ↑functional connectivity between left IFG and ACC during IC and between cerebellar activation and IFG and ACC during TT.	(Gilsbach et al., 2012)
NOS1	Exon 1f-VNTR #	fMRI	Reward anticipation task/ modified MID task	SS-carriers vs. SL/ LL-carriers	63 ADHD (38.3) 41 HC (38.0)	SS-carriers: ↑ in VS. No group x genotype interactions.	(Hoogman et al., 2011)
SLC6A3/ DAT1	3'UTR and intron 8 VNTR haplotype #	sMRI	Bilateral striatal volumes (nucleus accumbens, CN, and putamen)	Three DAT1 alleles (10/10 genotype, and the haplotypes 10-6 and 9-6)	118 ADHD (35.9) 111 HC (37) 301 ADHD (17.2) 186 HC (16.6) 1718 HC (26.1)	Adult ADHD 9-6 haplotype carriers ↑ 5.9% larger striatum volume relative to participants not carrying this haplotype (in adult ADHD patients only). Effect was not replicated in adolescent case-control and adult population-based cohort.	(Onnink et al., 2016)

Table 4: Continued

Gene	Variant	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
	3′ UTR VNTR#	sMRI	CN volume	9R-carriers vs. 10R/10R-carriers	33 ADHD (10.5) 26 HC (10.6)	9R-carriers: ↑ volumes of CN.	(Shook et al., 2011)
	3′UTR and intron 8 VNTR haplotype #	fMRI	VS and CN activity during reward-predicting cues	*SLC6A3* 10-6 dosage (2 copies vs. <2 copies)	29 ADHD (combined type; 15.8)‡ 30 HC (15.6)‡	ADHD: Activation in CN ↓ as number of copies ↑, but in control group reverse was found.	(Paloyelis et al., 2012)
			Striatal activity during reward anticipation task	9-6 haplotype carriers vs. non 9-6 haplotype carriers	87 ADHD (38.3) 77 HC (38)	No differences in striatal activity compared with non 9-6 haplotype carriers nor 9R- and 10R/10R-carriers.	(Hoogman et al., 2013)
				9-6 haplotype carriers vs. non 9-6 haplotype carriers	87 ADHD (38.3) 77 HC (38); same as above	Bayesian Constraint-based Causal Discovery (BCCD) algorithm confirmed that there is no evidence of a direct link between *DAT1* genetic variability and brain activation, but suggested an indirect link mediated through inattention symptoms and diagnostic status of ADHD	(Sokolova et al., 2015)
	3′ UTR VNTR#	fMRI	Working memory task	9R-carriers vs. 10R/10R-carriers	53 ADHD (35.7) 38 HC (31.2)	9R-carriers: ↓ left medial PFC activation compared to 10R/10R-carriers. Group × genotype interaction showed that 10R/10R-ADHD patients had ↑ activity in pre-SMA/dorsal ACC compared to HC.	(Brown et al., 2011)
			Go/No-go task	10R/10R carriers vs. 9R-carriers	20 ADHD (14.1) 38 HC (13.12)	10R/10R carriers: ↑ activity in frontal, medial, and parietal regions during response inhibition compared to 9R-carriers; ↓error response in the parahippocampal gyrus	(Braet et al., 2011)
				10R/10R carriers vs. 9R-carriers	33 ADHD (11.1)	10R/10R carriers: ↑ activity in left striatum, right dorsal premotor cortex, and temporoparietal cortical junction compared to 9R-carriers.	(Bedard et al., 2010)

Table 4: Continued

Gene	Variant	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
				9R-carriers vs. 10R/10R carriers	10 ADHD (14.6)‡ 10 unaffected siblings (14.8)‡ 9 HC (15.3)‡	9R-carriers: ↑ activity in CN and ↓ in cerebellar vermis compared to 10R/10R-carriers. Group × genotype interaction: effect in CN is observed in ADHD and unaffected siblings, but not HC.	(Durston et al., 2008)
			Multi-source interference task	10R/10R carriers vs. 9R-carriers	42 ADHD (35.4)	9R-carriers: ↓ activity in dorsal ACC compared to 10R/10R-carriers.	(Brown et al., 2010)
	3′ UTR VNTR	rs-fMRI	Striatal FC	9R/10R-carriers vs. 10R/10R carriers	50 HC (20.4)	9R/10R-carriers: stronger connectivity between dorsal CN and insula, dorsal anterior cingulate, and dorsolateral prefrontal regions, as well as between VS and ventrolateral PFC, compared with 10R/10R-carriers.	(Gordon et al., 2015)
		fMRI	Modified version of the MID task	10R/10R-carriers vs. 9R carriers	53 HC (29)	10R/10R-carriers: strong positive correlation between reward sensitivity and reward-related VS activity (relationship is absent in 9R-carriers).	(Hahn et al., 2011)
			Exposure to threatening faces	10R/10R-carriers vs. 9R-carriers	85 HC (45.2)	9R-carriers: ↑ amygdala reactivity compared with 10R/10R-carriers.	(Bergman et al., 2014)
			Go/No-Go task under influence of 40 mg MPH or placebo	9R-carriers vs. 10R/10R-carriers	50 HC (23.7)‡	9R-carriers: MPH induced ↑ activation during successful no-go trials compared with oddball trials in thalamocortical network 10R/10R-carriers: ↓ activation in thalamocortical network. Same pattern was observed in CN and IFG (successful no-go trials compared with successful go trials).	(Kasparbauer et al., 2015)
			Pre-cued task-switching task	9R-carriers vs. 10R/10R-carriers	20 HC (21.6)	9R-carriers: ↑ventromedial striatum activation during reward anticipation compared with 10R/10R-carriers; ↑ influence of anticipated reward on switch costs, and ↑activity in dorsomedial striatum during task switching in anticipation of high reward relative to low reward in 9R-carriers.	(Aarts et al., 2010)

Table 4: Continued

Gene	Variant	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
			Verbal n-back task	9R/10R-carriers vs. 10R/10R-carriers	20 HC (10.4)	9R/10R-carriers: ↑ performance accuracy, ↑ activation in frontal-striatal-parietal regions in high but not low runs compared with 10R/10R-carriers. Genotype x load interaction in right CN. 9R/10R-carriers: ↑ activation in striatal and parietal regions under high compared to low load, and genotype differences (9R/10R>10R/10R) were evident only under high load. 10R/10R-carriers: ↑ activation of substantial nigra/subthalamic nuclei under low than high load and genotype differences (10R/10R>9R/10R) were evident only under low load.	(Stollstorff et al., 2010)
SLC6A4/ 5HTT	5-HTTLPR #	sMRI (VBM)	GM volume	S-carriers vs. LL	291 ADHD 78 subthreshold ADHD 332 HC; Average age: 17 years	S-carriers: stress exposure is associated with ↓ GM volume in precentral gyrus, middle and superior frontal gyri, frontal pole, and cingulated gyrus. Association of G x E interaction with ADHD symptom count was mediated by GM volume in frontal pole and anterior cingulated gyrus only.	(van der Meer et al., 2015)
	5-HTTLPR	sMRI	Amygdala	SS vs. SL vs. LL	138 HC (41.2)	SS-carriers x anxiety: ↑ right amygdala volume (only in females)	(Cerasa et al., 2014)
			Hippocampus	S-carriers vs. LL	56 HC (71)	↓ Hippocampal volume in interaction with increased waking cortisol levels	(O'Hara et al., 2007)
				SS/SL vs. LL	357 HC (24.3)	S-carriers: ↓ hippocampal volume (females only); ↓ hippocampal volume correlated with severe CA (males only)	(Everaerd et al., 2012)
				S-carriers vs. LL	51 HC (~21)	↑ Left hippocampal volumes in woman ↓ Left hippocampal volumes in men	(Price et al., 2013)
				LL vs. SS/SL	159 HC (69.5)	LL-carriers x stress: ↓ hippocampal volume	(Zannas et al., 2013)

Table 4: Continued

Gene	Variant	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
			Multiple regions	S-carriers vs. LL	113 HC (37.6)	↓ GM volume of right IFG, left anterior cingulate, and superior temporal gyrus	(Selvaraj et al., 2011)
	5-HTTLPR, rs25531	sMRI	Total GM volume	SS vs. LL, S' vs. L'	58 HC (18.5)	No significant association with total GM volume	(Walsh et al., 2014)
	5-HTTLPR, rs25531, AluJb methylation of promoter	sMRI (VBM)	Hippocampus, amygdala, insula, anterior cingulated gyrus	S' vs. L' quantitative methylation score	Sample 1: 94 HC (36.9) Sample 2: 95 HC (34.2)	No significant association of genotype. Strong association of methylation and hippocampal GM volume; amygdala, insula, and CN showed similar associations, genotype-independent.	(Dannlowski et al., 2014)
	5-HTTLPR	sMRI (VBM)	GM volume	S-carriers vs. LL	sMRI: 114 HC (32.8) fMRI: 94 HC (31.3) (26 included in both)	S-carriers (VBM): ↓GM volume in limbic regions, particularly perigenual ACC and medial amygdala.	(Pezawas et al., 2005)
		fMRI	perceptual processing of fearful stimuli			S-carriers (fMRI): ↓ of amygdala- perigenual ACC connectivity, particularly in rostral ACC; ↓ structural covariance between amygdala and rostral ACC	
			GM volume, attentional interference task	S-carriers vs. LL	41 HC (adults)	S-carriers (VBM): ↑ volume in left cerebellum LL (VBM): ↑ volume in left superior and medial frontal gyri, left anterior cingulated, and right IFG S-carriers (fMRI): ↑ activation in response to negative, relative to neutral, words in right amygdala (driven by ↓ activation to neutral stimuli, rather than ↑ activation to negative stimuli); for negative-neutral contrast ↑ activation most prominent in insula, putamen, and CN	(Canli et al., 2005)

Table 4: Continued

Gene	Variant	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
		sMRI (VBM)	Hippocampus, amygdala	S-carriers vs. LL, interaction with SLEs	48 HC (24.7); 21 HC for perfusion scan	S-carriers: no correlation of hippocampus and amygdala volume with SLEs.	(Canli et al., 2006)
		fMRI	Face-stimuli			LL-carriers: positive correlation in GM volume with SLEs.	
		rs-fMRI	FC between amygdala and hippocampus; absolute CBF at rest			Negative correlation between SLEs and amygdala and hippocampus activation in response to face stimuli in S-carriers; positive correlation in LL-carriers. GxE effect altered FC between hippocampus and putamen. Interaction effect of 5-HTTLPR genotype and life stress on resting level activation in amygdala and hippocampus (positive correlation in S-group and negative correlation in L-group).	
		sMRI	GM volume	SS vs. LL	26 HC (20.3)	SS-carriers: No effect on amygdala and ventromedial PFC volume	(Rao et al., 2007)
		rs-fMRI	resting CBF			SS-carriers: ↑ resting CBF in amygdala and ↓ CBF in ventromedial PFC	
		DTI	WM integrity	L-carriers vs. SS	233 HC (22.7) §	L-carriers: ↓ anatomical connectivity between amygdala and PFC through uncinate fasciculus.	(Long et al., 2013)
		rs-fMRI	TC			L-carriers: ↓ FC between right amygdala and right frontal pole.	
	5-HTTLPR, rs25531	DTI	Structural connectivity	S'-carriers x SLE vs. L'L' x SLE	34 HC (25.6) †	↑ Structural connectivity between hippocampus and putamen (seed-based).	(Favaro et al., 2014)
		rs-fMRI	FC			↑ Positive correlation of co-activation of right parahippocampus and posterior cingulate cortex with SLEs (seed-based).	
	5-HTTLPR	rs-fMRI	Task-free activity	SS vs. LL	30 HC (20.3)	↑ Negative correlation of right amygdala activity and depressive symptoms	(Gillihan et al., 2011)

Table 4: Continued

Gene	Variant	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
			FC	SS vs. L-carriers	200 HC (22.1) ‡§	SS-carriers: ↑ fractional amplitude of low-frequency fluctuation in amygdala; ↓ rsFC between amygdala and various regions (including insula, Heschl's gyrus, lateral occipital cortex, superior temporal gyrus, hippocampus) and ↑ rsFC between amygdala and various regions (including supramarginal gyrus and middle frontal gyrus)	(Zhang et al., 2015a)
	5-HTTLPR, rs25531	rs-fMRI	FC	S'S' vs. S'L' vs. L'L'	39 HC (14.8)	↓ Superior medial frontal cortex connectivity ↓ Age-related increase in FC between posterior hub and superior medial frontal cortex	(Wiggins et al., 2012a)
	5-HTTLPR	fMRI	Sadness induction - regulation to normal emotion	SS vs. LL	30 HC (20.3)	↑ Amygdala activity during mood recovery.	(Gillihan et al., 2010)
			Emotion regulation task	S-carriers vs. LL	37 HC (22.6) †	↑ Right amygdala reactivity to fearful faces. ↑ Signal reductions in right amygdala during regulation of fear. ↑ Modulatory influence of cognitive regulation on FC between amygdala and bilateral ventrolateral PFC, left medial OFC, subgenual ACC and rostral ACC.	(Schardt et al., 2010)
				SS vs. LL	30 HC (20.3), same sample as above	↑Anti-correlation between amygdala and posterior cingulate cortex/precuneus during mood recovery.	(Fang et al., 2013)
	5-HTTLPR, rs25531	fMRI	Emotion regulation task	S'S' vs. L'L'	30 HC (20.5)	↓ Posterior insula and prefrontal brain activation during passive perception of negative emotional information. ↑ Prefrontal activation and anterior insula activation during down- and upregulation of negative emotional responses.	(Firk et al., 2013)
	5-HTTLPR	fMRI	Mood induction, sadness (film)	S-carriers vs. LL	48 HC (8.3)	↑ Right putamen, right CN, right rostro-ventral ACC, left CN, and left putamen in sad mood.	(Fortier et al., 2010)

Table 4: Continued

Gene	Variant	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
				S-carriers vs. LL	49 HC (12) †	↑ Earlier rise of left amygdala activation as sad mood increases.	(Furman et al., 2011b)
	5-HTTLPR	rs-fMRI	FC	LL vs. SS	38 HC (20.4) §	↑ Regional homogeneity in right amygdala; no effects on FC of right amygdala.	(Li et al., 2012)
		fMRI	Emotional processing			No difference in amygdala activity in response to negative stimuli.	
	5-HTTLPR, rs25531	fMRI	Emotion processing task	S'S' vs. S'L' vs. L'L' (treatment with escitalopram)	36 HC (25.1) †	↑Left amygdala activation with escitalopram treatment linearly related to 5-HTTLPR S' allele load for negative stimuli increased.	(Outhred et al., 2014)
	5-HTTLPR	fMRI	Emotional face task	S-carriers vs. LL	28 HC	S-carriers: ↑ right amygdala activity	(Hariri et al., 2002)
				S-carriers vs. LL	92 HC (30.5)	S-carriers: ↑ right amygdala activity	(Hariri et al., 2005)
				S-carriers vs. LL	29 HC (40) ‡	S-carriers: ↑ activation of amygdala and ↑ coupling between amygdala and ventromedial PFC.	(Heinz et al., 2005)
				SS vs. SL vs. LL	29 HC (37.5)	↑ Activity in right fusiform gyrus to fearful faces. ↑Positive FC between amygdala and fusiform gyrus and between right fusiform gyrus and right ventrolateral PFC.	(Surguladze et al., 2008)
				S-carriers vs. LL	21 HC (15)	↑ Left amygdala activation in response to anger.	(Battaglia et al., 2012)
	5-HTTLPR, rs25531	fMRI	Emotional face task	S'-carriers vs. L'L'	44 HC (30.3)	↑ Right amygdala responses to sad faces.	(Dannlowski et al., 2010)
				L'L' vs. S'S'	30 HC (26.6)	No association with amygdala reactivity. ↓ Subgenual cingulate cortex activation in response to fearful faces.	(O'Nions et al., 2011)

Table 4: Continued

Gene	Variant	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
		sMRI	Amygdala volume	S'-carriers vs. L'L'	54 HC (41.6)	↓ Amygdala volume Path analysis suggests effects on left amygdala volume are mediated by right amygdala volume but not through (midbrain) 5-HTT availability.	(Kobiella et al., 2011)
		PET	5-HTT availability			No genotype effect on (midbrain) 5-HTT availability.	
		fMRI	Amygdala activation			↑ Left amygdala activation in response to emotional stimuli.	
				S'S' vs. L'L	67 HC (18.6)	↑ Left amygdala reactivity in multivariate analysis; additive effects of recent SLEs.	(Walsh et al., 2012)
				S'S' vs. L'-carriers, interaction with SLEs	44 HC (26.8) ‡	↑ Bilateral amygdala activation in response to fearful faces. Interaction with SLEs: highest activity in S'S with SLEs for fearful faces in bilateral amygdala.	(Alexander et al., 2012)
		rs-fMRI		S'S' vs. L'-carriers	48 HC (14.8)	↓ Connectivity between right amygdala and ventromedial PFC with age.	(Wiggins et al., 2014a)
		fMRI				↑ Amygdala activation with age (age range 9-19 years)	
				S'-carriers vs. L'L' (bright-light intervention)	30 HC (24.3)‡	Bright-light dose positively associated with intra-prefrontal (medial PFC coupling with medial PFC seed) functional coupling only in S'-carriers.	(Fisher et al., 2014)
	5-HTTLPR	fMRI	Perceptual task of threatening stimuli	S-carriers vs. LL	14 HC phobic-prone (32.7) 14 HC eating disorders prone (34.3)	S-carriers: ↑ activity in right amygdala	(Bertolino et al., 2005)
		fMRI	Emotional face task with approach-avoidance	S-carriers vs. LL	48 HC (22.5) ‡	↑ Amygdala activity originating from reduced prefrontal inhibitory regulation.	(Volman et al., 2013)
			Emotional face-emotional word conflict task	S-carriers vs. LL	26 HC (70.5)	↓ Connectivity between dorsal ACC and pregenual ACC for incongruent face-word combination.	(Waring et al., 2014)

Table 4: Continued

Gene	Variant	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
	5-HTTLPR, rs25531	fMRI	Emotional face task with self-referential and emotion labeling conditions	S-carriers vs. LL, SLE interaction	45 HC (23.3)	↑ Amygdala activation and ↓ FC of amygdala with subgenual ACC in self-referential processing vs. emotion labeling. Negative correlation of bilateral amygdala activation during self-referential with SLEs in S-carriers; positive correlation in LL; pattern opposite during emotion labeling.	(Lemogne et al., 2011)
			Emotional face- word conflict task (Stroop-like task)	S'-carriers vs. L'L'	42 HC (~20)	↓ Recruitment of prefrontal control regions and superior temporal sulcus during conflict when task-irrelevant information was positively-valenced. ↑ Recruitment of these regions during conflict when task-irrelevant information was negatively-valenced.	(Stollstorff et al., 2013)
	5-HTTLPR	fMRI	Pain rating task	LL vs. SS	50 HC (24.9) †	↑ Positive linear effect of target pain in posterior cerebellum.	(Laursen et al., 2014)
			(un)predictable electric shocks	SS vs. L-carriers	51 HC (22) †	↑ Activity of amygdala, hippocampus, anterior insula, thalamus, pulvinar, CN, precuneus, ACC, and mPFC during threat anticipation. ↑ Positive coupling between mPFC activation and anxiety experience; L-carriers show ↑ negative coupling between insula and success of regulating anxiety.	(Drabant et al., 2012)
				S- carriers vs. LL	99 HC (21.9)‡ 69 HC (33.4)	S-carriers: ↑ dorsomedial PFC, anterior insula, bed nucleus of stria terminalis, thalamus and midbrain activation with increasing threat conditions across both samples.	(Klumpers et al., 2014)
	5-HTTLPR, rs25531	fMRI	Modified Flanker task	S'-carriers vs. L'L'	33 HC (23.4)	↑ Error-related rostral ACC activation. ↓ Conflict-related dorsal ACC activation.	(Holmes et al., 2010)

Table 4: Continued

Gene	Variant	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
			Decision making task	S'S' vs. L'L'	30 HC (26.6)	↑ Amygdala activation during decisions made counter to, relative to decisions made in accord with, the frame effect (gain or loss). Anterior cingulate-amygdala coupling during choices to made in counter to, relative to those made in accord with, the frame effect only observed in L'L'.	(Roiser et al., 2009)
			n-back task	S'S' vs. S'L' vs. L'L'	33 HC (37) †	↑ Bilateral prefrontal activation in right and left IFG pars triangularis with increasing S-allele count.	(Jonassen et al., 2012)
	5-HTTLPR	fMRI	Source memory task	S-carriers vs. LL	23 HC (66.8) [17 (23.3), not analyzed for genotype effects in fMRI]	↓ Activity in left IFG, middle frontal gyrus and anterior paracingulate cortex.	(Pacheco et al., 2012)
			Food / non-food pictures	LL vs. S-carriers	28 HC (25.5)	↑ Left posterior cingulate cortex activity for food pictures.	(Kaurijoki et al., 2008)
	5-HTTLPR, rs25531	fMRI	Differential fear conditioning	S'S' vs. L'-carriers	47 HC (26.8) ‡	↑ Activity in fear network: amygdala (right), insula, thalamus (left) and occipital cortex for conditioned stimulus. Interaction with SLEs: ↑ activity in right insula and left occipital cortex in S'S'.	(Klucken et al., 2013)

ACC = anterior cingulated cortex, ADHD = attention-deficit/hyperactivity disorder, BCCD = Bayesian Constraint-based Causal Discovery, BI = Behavioral Inhibition, BMI = Body mass index, BOLD = blood oxygen level–dependent, BPD = bipolar disorder, CA = childhood adversity, CBF = cerebral blood flow, CN = caudate nucleus, FC = functional connectivity, fMRI = functional magnetic resonance imaging, GM = gray matter, HC = healthy control, IC = Incompatibility Task, IFG = inferior frontal gyrus, MD = mean diffusivity, MID task = monetary incentive delay task, MPH = methylphenidate, OFC = orbitofrontal cortex, PET = positron emission tomography, PFC = prefrontal cortex, RCZ = rostral cingulate zone, rsFC = resting-state functional connectivity, SLE = stressful life events, SMA = supplementary motor area, sMRI = structural magnetic resonance imaging, TBV = total brain volume, TT = Time Discrimination Task, VBM = voxel-based morphometry, VS = ventral striatum, WM = white matter, † only females, ‡ only males; § Asian sample; # case-control studies; S'= functional S-allele (S or L_G), L'= functional L'-allele (L_A).

Imaging genetics of candidate genes for autism spectrum disorders

A total of 193 records were retrieved for the ASD search terms, and a total of six research articles were eligible for review according to our criteria. All studies investigated a single gene and were performed in Caucasian populations. For studies in the healthy population, we obtained 120 records, and 17 were included in the review (**Figure 1**). Twelve of those investigated a single gene in a Caucasian study sample, and five studies used Asian samples (studies for *SLC6A4/5HTT* are included in the ADHD section above). Generally, the ASD case/control samples included mainly childhood and adolescent study samples, whereas the studies in healthy population samples mostly used samples of (young) adults. From the eleven genes selected and listed in **Table 5**, imaging genetics studies could only be retrieved for genetic variants in *CNTNAP2*, *MET*, *OXTR*, and the *SLC6A4/5HTT* gene.

The **contactin-associated protein-like 2** (CASPR2), encoded by the gene *CNTNAP2* (the largest gene in the human genome), is a neural transmembrane protein involved in neuronal-glial interactions and in clustering K^+-channels in myelinated axons; as such, it is involved in neuronal cell adhesion, migration, and the formation of neuronal networks (Rodenas-Cuadrado et al., 2014). Several single nucleotide polymorphisms (SNPs) in *CNTNAP2* have been associated with ASDs. During human brain development, *CNTNAP2* expression is broad, with highest levels in frontal and anterior lobes, striatum, and dorsal thalamus. This cortico-striato-thalamic circuitry is important for higher order cognitive functions, including speech and language, reward, and frontal executive function (Rodenas-Cuadrado et al., 2014). This is reflected in the imaging genetics studies having been performed for *CNTNAP2*, which cover studies of brain volume and structural connectivity as well as brain activity and functional connectivity during tasks related to rewarded learning and language (**Table 5**).

Two studies performed DTI in healthy individuals. For the SNP rs2710102 it was found that carriers of the CC risk genotype showed reduced overall path length and increased small-worldness of brain-wide structural connectivity, which appeared to be a general phenomenon rather than being localized to individual tracts (Dennis et al., 2011). A large study in healthy individuals combining sMRI with DTI for the SNP rs7794745 showed that carriers of the ASD risk genotype exhibited reduced gray and white matter volume as well as reduced white matter integrity in the cerebellum, fusiform gyrus, occipital and frontal cortices; distribution of reductions was found to be sex-specific (Tan et al., 2010).

In a case-control study, an association between the SNP rs2710102 and medial prefrontal cortex activation during a rewarded implicit learning task was found, when collapsing patients and controls into one group. The non-risk allele was linked to reduced activation. Furthermore, the risk carriers had more widespread and bilateral connectivity throughout the frontal cortex and anterior temporal poles. The latter finding was confirmed in an independent healthy sample (Scott-Van Zeeland et al., 2010). An additional fMRI study using a sentence completion paradigm showed that carriers of the risk genotype for one of two

SNPs had increased activation of the IFG (Broca's area), the lateral temporal cortex, or right middle temporal gyrus (Whalley et al., 2011).

The **Met proto-oncogene** encoded by the *MET* gene is a cell surface receptor with tyrosine-kinase activity. In the forebrain, *MET* gene and protein expression is regulated in excitatory projection neurons during synaptogenesis (Judson et al., 2011) and is restricted to regions of temporal, occipital, and medial parietal cortex in humans. These regions are known to be of relevance to the processing of socially relevant information (Rudie et al., 2012). The effects of the ASD risk variant rs1858830 have been studied in two imaging genetics studies (**Table 5**).

A case-control study combining fMRI (emotional face task), resting-state fMRI, and DTI modalities showed that the ASD risk genotype predicted wide-spread atypical brain activity patterns to social stimuli, with increased activation in amygdala and striatum, and impaired deactivation patterns in part of the default mode network (DMN) in the posterior cingulate cortex. In addition, reduced functional and structural connectivity was observed in temporo-parietal regions belonging to the DMN suggesting altered white matter integrity. In general, the effects were more pronounced in the ASD group (Rudie et al., 2012). An sMRI study in a large sample of healthy individuals revealed that cortical thickness in temporal, pre- and postcentral gyri, anterior cingulate, and frontopolar cortex was reduced in risk-allele carriers, with reductions increasing with increasing number of risk alleles (Hedrick et al., 2012).

The **oxytocin receptor** (*OXTR*) gene encodes the receptor protein for oxytocin, which has an important role in the regulation of social cognition and behavior (Meyer-Lindenberg et al., 2011). So far, no imaging genetic studies were performed for risk variants in the *OXTR* gene in ASD case-control samples, but twelve studies in healthy samples were found (**Table 5**). Various different SNPs and combinations of those were investigated, not all related to ASD risk.

Two sMRI studies showed that adolescents homozygous for the rs2254298 risk factor for psychopathology displayed an overall increased gray matter volume, but a decreased amygdala volume (Furman et al., 2011a); for carriers of the rs53576 SNP, a risk factor for disorders associated with social impairment, a smaller hypothalamus gray matter volume was reported in healthy adults (Tost et al., 2010).

Functional MRI paradigms used to study *OXTR* all covered the cognitive domains of emotion processing and reward (**Table 5**). In a face matching task, adult carriers of the rs53576 risk allele showed increased functional correlation of hypothalamus and amygdala during perceptual processing of facial emotion (Tost et al., 2010). Investigating a large group of 1445 healthy adolescents in a passive face viewing task for effects of 23 SNPs across *OXTR*, the IMAGEN Consortium found significant effects of one SNP on ventral striatal activity in a region of interest analysis. In the presence of stressful life events, this SNP modulated the occurrence of emotional problems in the participants, linking more

emotional problems to reduced striatal activation; no effects of the risk variants for ASD were observed (Loth et al., 2014). A study of brain regions related to processing of social stimuli observed increased functional connectivity between such regions in adult carriers of the risk genotype for rs53576 (Verbeke et al., 2013). Functional MRI of mesolimbic structures during reward processing was modulated by the rs2268493 risk factor for ASD: young adult carriers of the risk genotype showed reduced activation in mesolimbic reward circuitry (nucleus accumbens, amygdala, insula, thalamus, and prefrontal cortical regions) during the anticipation of rewards but not during reward receipt (Damiano et al., 2014). Using a mother-child interaction task, Michalska and coworkers (2014) showed that females carrying the ASD risk genotypes for rs53576 or rs1042778 had lower brain activity in OFC, ACC, and hippocampus in response to child stimuli. When healthy adult females were tested for empathic response and associated brain activation, carriers of the rs2254298 risk factor for psychopathology showed increased responsiveness of the superior temporal sulcus to observed pain (Laursen et al., 2014). In a pharmacologic imaging genetics study in adult males, one of three SNPs modulated the response of the amygdala (only) after oxytocin inhalation, with increased activation to directed gaze and decreased activation to averted gaze under oxytocin in the carriers of the variant allele (Montag et al., 2013). This study did not find any effects of rs2254298 on brain activation.

In summary, genetic variation in the *OXTR* gene has been linked to brain activation during emotional processing. Risk factors for ASD/psychopathology appear to reduce activation during most relevant paradigms, but may increase functional connectivity during those tasks.

Four ASD case-control imaging genetics studies investigated the gene encoding the **serotonin transporter** gene (*SLC6A4*, *5HTT*) in addition to those in healthy individuals (and ADHD case-control samples) described in the section on ADHD candidate genes. Structural MRI, fMRI, and rs-fMRI were used to study the effect of either only the 5HTTLPR or the combination of this variant with rs25531 (**Table 5**).

Whereas a VBM study did not reveal an association between total gray or white matter volume and genotype in adult patients (Raznahan et al., 2009), another sMRI study showed that in 2-4 year old boys with ASD, carriers of the 5HTTLPR S-allele had increased total cortical and frontal lobe gray matter volume (Wassink et al., 2007), suggesting an age-dependent effect of the variant.

The fMRI and rs-fMRI study, performed in overlapping samples of adolescent patients and controls, showed that carriers of alleles that mark low gene expression had increased amygdala activation during an emotional face task, an effect that was observed only in the patients (Wiggins et al., 2014b), and increased posterior-anterior connectivity during a resting-state condition in patients, where the converse was observed in the healthy group (Wiggins et al., 2012b).

The findings of those case-control studies are not easily reconciled with those observed in healthy individuals (**Table 4** and **6**), and indeed the latter two studies suggest the existence of differential effects in patients and healthy individuals.

Table 5: Imaging genetics studies in ASDs case-control samples and ASDs candidate genes studies in the healthy population (for selection of candidate genes see **Table 2**).

Gene	Polymorphism	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
CNTNAP2	rs2710102	fMRI	Reward-guided implicit learning task (fronto-striatal circuits)	C-allele carriers vs. non-risk-carriers	Discovery sample: 16 ASD (12.4) ‡ 16 HC (12.3) ‡	Non-risk group (collapsed across patients and controls): ↓ Activity in medial PFC during reward feedback processing; Risk group: ↑ long-range anterior-posterior connectivity between medial PFC, medial occipital, and ventral temporal cortices.	(Scott-Van Zeeland et al., 2010)
				C-allele carriers vs. non-risk-carriers	Replication sample: 39 HC (13)	Non-risk-group: ↑ long-range anterior-posterior functional connectivity between mPFC, medial occipital, and ventral temporal cortices.	
	rs2710102	DTI	Whole-brain fiber tractography (graph theory analyses)	CC-carriers vs. CT/TT-carriers	328 HC (23.4); twins from 189 families	CC-carriers: ↓ path length, ↑ small-worldness and global efficiency in whole-brain analyses, and ↓ eccentricity (maximum path length) in 60 of the 70 nodes in regional analyses.	(Dennis et al., 2011)
	rs7794745	sMRI	WM and GM morphology	TT-carriers vs. AT/AA-carriers	314 HC	TT-carriers: ↓ GM and WM volume in cerebellum, fusiform gyrus, occipital and frontal cortices. Male TT-carriers: ↓ GM in right frontal pole in right rostral fronto-occipital fasciculus.	(Tan et al., 2010)
		DTI	WM integrity			TT-carriers: ↓ FA in cerebellum, fusiform gyrus, occipital and frontal cortices. Male TT-carriers: ↓FA in right rostral fronto-occipital fasciculus. Female TT-carriers: ↓ FA of anterior thalamic radiation.	
	rs7794745, rs2710102	fMRI	Language task	Risk group (T- and C-allele) vs. non-risk group	66 HC (20.54)	Risk group: ↑ activation in right IFG (Broca's area homologue) and right lateral temporal cortex.	(Whalley et al., 2011)

Table 5: Continued

Gene	Polymorphism	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
MET	rs1858830	fMRI	Emotional faces task (n = 144), DMN functional connectivity (n = 71), WM structural connectivity (n = 84).	CC-carriers vs. CG-carriers vs. GG-carriers (non-risk)	75 ASD (13.1) 87 HC (12.5)	Risk genotype predicted wide-spread atypical fMRI activation (↑ amygdala and striatum) and deactivation patterns (↓ mainly posterior cingulate cortex) to social stimuli. Effects were more pronounced ASD group, especially within heterozygous risk group.	(Rudie et al., 2012)
		rs-fMRI				Risk genotype: ↓ Functional and structural connectivity in temporo-parietal regions (within DMN)	
		DTI				Risk genotype: Altered WM integrity	
	rs1858830	sMRI	Measures of cortical thickness (CT) development	CC-carriers vs. CG-carriers vs. GG-carriers	222 HC (9-22)	C-carriers: ↓ CT (lowest in CC group) in superior and middle temporal gyri, ventral precentral and postcentral gyri, and anterior cingulate bilaterally, and in right frontopolar cortex.	(Hedrick et al., 2012)
OXTR	rs2254298	sMRI	Amygdala volume, TBV	GG-carriers vs. GA-carriers	51 HC (13) †	GG-carriers: ↑ GM volume, ↓ amygdala volumes. VBM analysis revealed ↑ volume in region of dorsomedial ACC in GG-carriers and ↑ in posterior brainstem in G/A-carriers	(Furman et al., 2011a)
		sMRI (VBM)	Global brain measures (GM, WM, TBV)	AA- carriers vs. AG-carriers vs. GG-carriers	135 HC (28.8)§	Male A-allele carrier: ↓ GM volume in right insula (neuroanatomical correlate of ALTs).	(Saito et al., 2014)
	rs1042778, rs2254298, rs237887, rs918316, rs2268493, rs53576, rs2268495	sMRI	Amygdala and hippocampus volume, TBV	rs2254298: AA- carriers vs. AG-carriers vs. GG-carriers	208 HC (33.9)§	rs2254298: A-allele carriers: ↑ bilateral amygdala volume. Two 3-SNP haplotypes (including rs2254298 G-allele), showed associations with ↓ bilateral amygdala volume.	(Inoue et al., 2010)

Table 5: Continued

Gene	Polymorphism	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
	rs53576	sMRI (VBM)	Global brain measures (GM, WM, TBV)	AA-carriers vs. GG/GA-carriers	290 HC (23.7)§	Female AA-carriers: ↓ amygdala volumes bilaterally (especially centromedial subregion, with a trend of allele-load-dependence)	(Wang et al., 2014a)
		rs-fMRI	rsFC			↓ Resting-state functional coupling between PFC and amygdala bilaterally (allele-load-dependent trend).	
		rs-fMRI	Functional connectivity density (FCD) using a voxel-wise, data-driven approach	Male AA-carriers vs. male G-allele carriers	270 HC (24.2)§	FCD of hypothalamus exhibited main effect of genotype (↓FCD in male AA homozygotes). Gender-by-genotype interaction in resting-state FC (rsFC) between hypothalamic region and left dorsolateral PFC, but no main effect of genotype (↓ rsFC in male AA homozygotes).	(Wang et al., 2013)
		sMRI (VBM)	Regional alterations in GM volume	GG-carriers vs. GA-carriers vs. AA-carriers	VBM: 212 HC (29.9) fMRI: 228 HC (31.9) (98 overlap)	A-allele carriers: ↓ hypothalamus GM volume	(Tost et al., 2010)
		fMRI	Face-matching task			A-allele carriers: ↓ amygdala activation, ↑ functional correlation of hypothalamus and amygdala during perceptual processing of facial emotion (specifically in male risk allele carriers lower levels of reward dependence predicted).	
	23-tagging SNPs (including rs7632287, rs237887, rs2268491, rs2254298, rs2268494)	fMRI	Animated angry faces task	rs237915: CC-carriers vs. CT/TT-carriers	1445 HC (14.4)	CC-carriers: ↓ VS activity (related to more peer problems).	(Loth et al., 2014)

Table 5: Continued

Gene	Polymorphism	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
	rs53576	fMRI	Others' suffering task	GG-carriers vs. AA-carriers	60 HC (20.2)§	GG-carriers: hierarchical regression analyses revealed ↑ associations between interdependence and empathic neural responses in insula, amygdala, and superior temporal gyrus.	(Luo et al., 2015)
		fMRI	Emotional-valenced stimuli task	GG-carriers vs. AG/AA-carriers	21 HC (34)	GG-carriers: ↑ functional connectivity between regions of interest. Bilateral amygdala and medial PFC show ↑ influence on other brain regions; bilateral pars opercularis, left amygdala, and left medial PFC are more receptive to activity in other brain regions.	(Verbeke et al., 2013)
	rs1042778, rs2268493, rs237887	fMRI	MID task	rs2268493: TT-carriers vs. CT/CC-carriers	31 HC (23.6)	rs2268493 TT-carriers: ↓ Activation in mesolimbic reward circuitry (nucleus accumbens, amygdala, insula, thalamus and prefrontal cortical regions) during anticipation of rewards but not during outcome phase.	(Damiano et al., 2014)
	rs53576, rs1042778	fMRI	Mother-child interaction task	3 genotype groups per SNP	40 HC †	Both rs53576 and rs1042778 were associated with both positive parenting and hemodynamic responses to child stimuli in OFC, ACC, and hippocampus (rs53576 GG group showed lowest hemodynamic response).	(Michalska et al., 2014)
	rs2268498, rs180789, rs401015	fMRI, double-blind placebo-controlled crossover study	Social–emotional and gaze processing task; amygdala activation after intranasal oxytocin self-administration	rs401015: CT-carriers vs. TT-carriers	55 HC (24.9) ‡	rs401015 modulated right amygdala activity under influence of oxytocin (CT-carriers: ↑ amygdala activity).	(Montag et al., 2013)

Table 5: Continued

Gene	Polymorphism	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
SLC6A4*/ *5HTT	5-HTTLPR	sMRI (VBM)	Total GM and WM volume	LL vs. LS vs. SS	43 ASD (30)	No associations between total GM or WM volume and genotype.	(Raznahan et al., 2009)
		sMRI (longitudinal)	Cerebral cortical and cerebellar GM and WM volume	SS vs. SL vs. LL	44 ASD (3.4) ‡	S-carriers: ↑ cortical and frontal lobe GM	(Wassink et al., 2007)
	5-HTTLPR, rs25531	rs-fMRI	Functional connectivity	Low vs. high expressing	54 ASD (13.7) 66 HC (14.5)	Low expressing genotypes (SS, SL_G, L_GL_G): ↑ posterior-anterior connectivity in ASD group (converse for HC).	(Wiggins et al., 2012b)
		fMRI	Emotional faces task	Low vs. high expressing	44 ASD (13.5) 65 HC (14.7)	Low expressing genotypes (SS, SL_G, L_GL_G): ↑ amygdala activation in ASD group.	(Wiggins et al., 2014b)

ACC = anterior cingulate cortex, ALT = autistic-like traits, CA = childhood adversity, CN = caudate nucleus, CT = cortical thickness, CV = cortical volume, DMN = default mode network, DTI = diffusion tensor imaging, GM = gray matter, FC = functional connectivity, FCD = functional connectivity density, HC = healthy control, IFG = inferior frontal gyrus, MID = monetary incentive delay task, mPFC = medial prefrontal cortex, OFC = orbitofrontal cortex, PFC = prefrontal cortex, ROI = region of interest, rsFC = resting-state functional connectivity, SA = surface area, SLE = stressful life events, sMRI = structural magnetic resonance imaging, STS = superior temporal sulcus, VBM = voxel-based morphometry, VS = ventral striatum, WM = white matter, TBV = total brain volume, † only females, ‡ only males; § Asian sample; in gray only case-control studies; for *SLC6A4* studies in healthy individuals see Tables 4 and 6 (ADHD).

Table 6: Imaging genetics studies in ADHD and ASDs case-control samples and candidate genes studies in the healthy population studying more than one single gene.

ADHD/ASD candidate gene (polymorphism)	Additional gene(s) studied	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared (candidate genes or interaction)	Samples size (mean age in years)	Primary results (main effect of candidate gene genotype or interaction)	Reference
SLC6A3 (3′ UTR VNTR), ***DRD4*** (exon 3 VNTR)	---	sMRI	PFC gray matter and CN volume	9R-carriers vs. 10R/10R-carriers, 4R/4R-carriers vs. rest	26 ADHD (12.1) 26 unaffected siblings (11.6) 20 HC (10.7); all ‡	*SLC6A3* 9R-carriers: ↑ CN volumes *DRD4* 4R/4R-carriers: ↓ prefrontal GM volume. No effects on CN, or TBV. No interactions between ADHD status and genotype.	(Durston et al., 2005)
	DRD1	sMRI; longitudinal study (mean follow-up, 6 years)	Cortical thickness	9R-carriers vs. 10R/10R-carriers, 7R-carriers vs. non-7R-carriers	105 ADHD (10.1; 13.1; 15.9) 103 HC (10.0; 12.4; 14.4)	*SLC6A3* 9R-carriers: No effect on cortical development. *DRD4* 7R-carriers: thinner right orbitofrontal/ inferior prefrontal and posterior parietal cortex. ADHD 7R-carriers: distinct trajectory of cortical development; normalization of right parietal cortical region.	(Shaw et al., 2007)
	COMT	DTI	WM integrity, FA values	9R-carriers vs. 10R/10R-carriers; 4R/4R-carriers vs. rest	58 stimulant- and atomoxe-tine-naïve ADHD (8.7) §	*SLC6A3* 9R-carriers: no effect on WM integrity *DRD4* 4R/4R-carriers: no effect on WM integrity.	(Hong et al., 2015)
SLC6A3 (3′ UTR VNTR)	*COMT*	fMRI	Episodic memory task	9R-carriers vs. 10R/10R-carriers	49 HC (22.7)	9R-carriers: ↑ midbrain activation (right substantia nigra and the ventral tegmental area)	(Schott et al., 2006)
			N-back task	9R/9R-carriers x val/ val-carriers	75 HC (19.6)	No effects on brain activation were found for each genotype independently. Val/val and 9R/9R subjects show highest activation dorsolateral prefrontal region.	(Caldu et al., 2007)
			Response inhibition (stop-signal) task	9R-carriers vs. 10R/10R-carriers	43 HC (22.7)	*SLC6A3* 9-allele carriers: ↑ activation during inhibition in subthalamic nucleus and (pre-) supplementary motor area	(Congdon et al., 2009)

Table 6: Continued.

ADHD/ASD candidate gene (polymorphism)	Additional gene(s) studied	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared (candidate genes or interaction)	Samples size (mean age in years)	Primary results (main effect of candidate gene genotype or interaction)	Reference
			Reward anticipation task	9R-carriers vs. 10R/10R-carriers; val-carriers vs. met/met-carriers	22 HC (27.9)	*SLC6A3* 9R- carriers: highest activity in CN and VS during reward anticipation and in lateral PFC and midbrain at time of reward delivery. Interaction *SLC6A3* and *COMT*: *DAT1* 9R-allele carriers and *COMT* met/met-allele carriers showing highest activation in VS and lateral PFC during reward anticipation and in lateral prefrontal and orbitofrontal cortices, and in midbrain at time of reward delivery.	(Dreher et al., 2009)
	TREK, *COMT*	fMRI	MID task	9R-carriers vs. 10R/10R-carriers	32 HC (21.7)	*TREK1* and *SLC6A3* /*COMT* genotypes were independently related to basal ganglia responses to gains.	(Dillon et al., 2010)
	COMT	fMRI	Fear conditioning, extinction and reacquisition task	9R- carriers vs. 10R/10R-carriers	69 HC ‡	9R- carriers: ↑ learning rates and stronger hemodynamic appetitive prediction error signals in VS.	(Raczka et al., 2011)
SLC6A4 (5-HTTLPR)	*BDNF*	sMRI	Global GM volume	S-carriers x val/val	111 HC (32.60)	↓ ACC volume	(Pezawas et al., 2008)
SLC6A4 (5-HTTLPR, rs25531, STin2)	*OXTR*, *STMN1*	sMRI	Amygdala volume	SS vs. SL vs. LL.	139 HC (22)‡	*SLC6A4* risk alleles are associated with ↓ amygdala volumes.	(Stjepanovic et al., 2013)
SLC6A4 (5-HTTLPR)	*COMT*	sMRI (VBM)	Global GM volume	S-carriers vs. LL-carriers x met-carriers vs. val/val-carriers	91 HC (33)	Interaction: ↓GM volume of bilateral parahippocampal gyrus, amygdala, hippocampus, vermis of cerebellum and right putamen/insula	(Radua et al., 2014)
SLC6A4 (5-HTTLPR, rs25531)	*TPH2*	fMRI	MID task	L'L' vs. S'-carriers	89 HC (27.8)	L'L'-carriers: positive association with amygdala-hippocampus activity and trait anxiety score.	(Hahn et al., 2013)

Table 6: Continued.

ADHD/ASD candidate gene (polymorphism)	Additional gene(s) studied	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared (candidate genes or interaction)	Samples size (mean age in years)	Primary results (main effect of candidate gene genotype or interaction)	Reference
SLC6A4 (5-HTTLPR)	*MAOA*	fMRI	Response inhibition task	S-carriers vs. LL	35 HC (32.1) ‡	S-carriers: ↑ activation in ACC Allele–allele interaction: ↑ BOLD activity in ACC.	(Passamonti et al., 2008)
SLC6A4 (5-HTTLPR, rs25531)	*COMT*	fMRI	Emotion processing task	S'S'-carriers and L'L'-carriers x val/val –carriers and met/met-carriers	48 HC (41.2) ‡	Interaction effects in amygdala, hippocampal and limbic cortical regions elected by unpleasant stimuli. No additive or interaction effects.	(Smolka et al., 2007)
			Emotional face task	S'-carriers vs. L'L' met/met vs. val-carrier s	54 HC (24.1)	S'-carriers : ↑right amygdala activity in response to angry stimuli.	(Lonsdorf et al., 2011)
SLC6A4 (5-HTTLPR)	*TPH2, HTR1A, HTR2A*	fMRI	Emotional face task	L-carriers vs. SS	55 HC (23.3) †§	L-carriers: ↑Bilateral amygdala activation in response to angry faces	(Lee and Ham, 2008)
SLC6A4 (5-HTTLPR, rs25531)	*COMT*	fMRI	Emotional face task	S'S'-carriers and L'L'-carriers x met/met-carriers and val/val-carriers	91 HC (32.5)	Interaction: ↓Reciprocal connectivity within bilateral fusiform and inferior occipital regions, right superior temporal gyrus and superior temporal sulcus, bilateral inferior and middle PFC and right amygdala, in fear processing conditions.	(Surguladze et al., 2012)
SLC6A4 (5-HTTLPR)	*TPH2*	fMRI	Emotional face task	S-carriers and LL-carriers x *TPH2*	49 HC (24.0)	Interaction: ↑ activation of putamen and amygdala, most robust for visuospatial and negatively valenced stimuli	(Canli et al., 2008)
SLC6A4 (5-HTTLPR, rs25531)	*BDNF*	fMRI	Emotion processing	S-carriers vs. LL; interaction val/met	28 HC (24.49)†	S-carriers: ↑rostral ACC and amygdala activation during presentation of emotional images. S-carriers and met-carriers: ↑ activation in rostral ACC and amygdala.	(Outhred et al., 2012)

Table 6: Continued.

ADHD/ASD candidate gene (polymorphism)	Additional gene(s) studied	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared (candidate genes or interaction)	Samples size (mean age in years)	Primary results (main effect of candidate gene genotype or interaction)	Reference
DRD2 (A1 allele)	*BDNF*	sMRI	Global GMV	A1-carriers x met-carriers	161 HC (27.29)	Interaction: ↓GM volume of ACC	(Montag et al., 2010)
DRD4 (rs1800955)	*COMT*	fMRI	Gambling paradigm featuring unexpectedly high monetary gains and losses	CC-carriers vs. TT-carriers	53 HC (21.2)	CC-carriers: ↑ responses in anterior insula and cingulate cortex.	(Camara et al., 2010)
DRD2 (rs1800497), ***DRD4*** (exon 3 VNTR)	---	fMRI	Imagined intake of palatable foods, unpalatable foods, glasses of water (pictures).	A1-carriers and 7R-carriers	44 HC (15.6) †	↓ Activation of frontal operculum, lateral OFC, and striatum in response to imagined intake of palatable foods (vs. unpalatable food or water), predicted future ↑ in body mass for those with A1 or 7R-allele.	(Stice et al., 2010)
SLC6A3 (3' UTR VNTR), ***DRD2*** (rs1800497)	*COMT*	fMRI	Cue-target reading paradigm	A1-carriers vs. A2/A2, 9R-carriers vs. 10R/10R, met/met vs. val/met vs. val/val	71 HC (27.6) ‡	*DRD2* polymorphism did not affect results. 10R-carriers: ↑ dorsal IFG activation. Linear effect of *COMT* val/met and *DAT1* 9R/10R on preparatory activity in left IFG pointed to negative interaction between tonic lateral prefrontal and phasic subcortical DA.	(Arnold et al., 2015)

Table 6: Continued.

ADHD/ASD candidate gene (polymorphism)	Additional gene(s) studied	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared (candidate genes or interaction)	Samples size (mean age in years)	Primary results (main effect of candidate gene genotype or interaction)	Reference
DRD2 (rs1800497), ***DRD4*** (exon 3 VNTR), ***SLC6A3*** (3' UTR VNTR and intron 8 VNTR)	*ADRA1A, ADRA1B, ADRA1D, ADRA2A, ADRA2B, ADRB1, ADRB2, ADRB3, COMT, DBH, DDC, DRD1, DRD3, DRD5, SLC6A2, TH*	fMRI	Stop-signal task	*SLC6A3* rs37020 (T-carriers vs. GG-carriers)	50 HC (22.1)	Activity in frontal regions (anterior frontal, superior frontal and superior medial gyri) and CN varied additively with T-allele of rs37020.	(Cummins et al., 2012)
DRD2 (rs1800497, rs1799732), ***DRD4 (***exon 3 VNTR) ***SLC6A3*** (3' UTR VNTR)	*COMT*	fMRI	Receipt and anticipated receipt of palatable food and monetary reward	Individual risk genotypes and multilocus score	160 HC (15.3)	Individuals with 5 'risk' genotypes: did not show ↓ activation of DA-based reward regions. *DRD4*-L vs. *DRD4*-S genotype: ↓ middle occipital gyrus activation in response to monetary reward. Multilocus composite score: ↑ number of 'risk' genotypes ↓ activation in putamen, CN, and insula in response to monetary reward.	(Stice et al., 2012)

Table 6: Continued.

ADHD/ASD candidate gene (polymorphism)	Additional gene(s) studied	Imaging modality	Imaging/cognitive phenotype	Genotype groups compared (candidate genes or interaction)	Samples size (mean age in years)	Primary results (main effect of candidate gene genotype or interaction)	Reference
			Card guessing game task	Multilocus DA profile	69 HC (44.5)	↑ Reactivity correlated with ↑ number of risk factors. Multilocus DA profile scores accounted for 10.9% of inter-individual variability in reward-related VS reactivity. None of individual polymorphisms accounted for significant variability.	(Nikolova et al., 2011)
SLC6A4 (5-HTTLPR, rs25531), ***OXTR*** (rs2268498 and rs53576)	---	fMRI	Empathic performance task (facial responses of target person to electric stimulation)	SS-carriers vs. LL-carriers; rs2268498: CC- vs. CT- vs. TT-carriers; rs53576: AA-vs. AG-vs. GG-carriers	50 HC (24.9) †	rs2268498 CC-carriers: high empathic accuracy was associated with ↑ responsiveness of right STS to observed pain.	(Laursen et al., 2014)

ACC = anterior cingulate cortex, ADHD = Attention deficit/hyperactivity disorder, BOLD = blood oxygen level–dependent, CN = caudate nucleus, DA = dopamine, DTI = diffusion tensor imaging, FA = fractional anisotropy, fMRI = functional magnetic resonance imaging, GMV = gray matter volume, HC = healthy control, MID task = monetary incentive delay task, OFC = orbitofrontal cortex, PFC = prefrontal cortex, sMRI = structural magnetic resonance imaging, UTR = untranslated region, TBV = total brain volume, VAC task = variable attentional control task, VNTR = variable number tandem repeat, VS = ventral striatum, VSWM = visuospatial working memory, WM = white matter, † only females, ‡ only males, § Asian sample; in gray only case-control studies.

Imaging genetics in selected intellectual disability disorders

A total of 579 records were retrieved for the ID syndromes of interest. Eighty research articles were eligible for review according to our criteria, 30 for fragile X syndrome, 24 for neurofibromatosis type 1, 22 for tuberous sclerosis complex, and four for Rett syndrome (**Figure 1**). No imaging studies of Timothy syndrome patients were uncovered by our search term. The reviewed imaging genetics studies in ID syndromes are presented in **Table 7**.

The **fragile X mental retardation 1** gene (*FMR1*) is located on the X chromosome and codes for fragile X mental retardation protein. Large expansions of a CGG repeat (>200 repeats) in the 5'- untranslated (5'UTR) region of the gene, leading to protein deficiency, are the cause of fragile X syndrome (FXS). FMR1 has a prominent role in synaptic plasticity and maturation (Saldarriaga et al., 2014). In studies including participants with the *FMR1* full mutation, brain structure was most often investigated, followed by task-based brain activation (**Table 7**). A few studies investigated brain structural integrity and resting-state functional connectivity. Several studies compared individuals with FXS with and without ASD or included an idiopathic autism or IQ-matched group (**Table 7**).

The most robust finding in investigations of brain structure in FXS is an increased caudate nucleus volume. This enlargement was observed early in development (Hazlett et al., 2009), throughout adolescence (Bray et al., 2011; Hall et al., 2013; Lee et al., 2007) as well as in adult samples (Hallahan et al., 2011; Molnar and Keri, 2014; Wilson et al., 2009). Studies comparing individuals with FXS and with ASD found increased caudate volumes in children and adults with FXS compared to children/adults with idiopathic autism (Hazlett et al., 2009; Wilson et al., 2009). Consistent volumetric abnormalities have also been found for cerebellar regions in FXS; a reduction in the volume was observed in both children and adults with FXS (Hazlett et al., 2012; Hoeft et al., 2008; Wilson et al., 2009). Several studies found cerebellar volumes to be larger in children and adults with FXS relative to individuals with autism, in whom reduced volume of cerebellar regions compared to control subjects is often seen as well (Hazlett et al., 2012; Wilson et al., 2009). Few studies have investigated white matter integrity in people with the full *FMR1* mutation, and deficits seem most prominent in fronto-striatal connections. Increased density of fibers was found in the left ventral fronto-striatal pathway in boys with FXS compared to typically developing and developmentally delayed controls (Haas et al., 2009), and differences in white matter in frontal-caudate circuits were found in females with FXS compared to controls (Barnea-Goraly et al., 2003). More widespread reductions in white matter integrity have also been observed (Villalon-Reina et al., 2013).

Cognitive and psychiatric characteristics associated with FXS include poor eye contact, repetitive motor behavior, language deficits, inattention, hyperactivity, inhibition, and anxiety (Saldarriaga et al., 2014). Functional neuroimaging studies have focused on these deficits, with a main focus on poor eye contact and behavioral inhibition. Several fMRI studies have investigated the circuitry underlying face/gaze processing in subjects with FXS, as eye-gaze avoidance is common in this population. Abnormal activation was found

in several regions, including superior temporal gyrus and fusiform gyrus (Garrett et al., 2004), amygdala and insula (Watson et al., 2008), regions within the ventrolateral prefrontal cortex (vlPFC) (Holsen et al., 2008), and frontal cortex and cingulate and fusiform gyri (Bruno et al., 2014). These regions are associated with visual processing, social cognition, emotion processing, and executive functioning, indicating that eye-gaze avoidance in FXS may be linked to social anxiety. Investigating attention and inhibition, a study using a Go/No-go task found that boys with FXS show reduced activation in the right vlPFC and caudate head. The authors suggested that defective fronto-striatal signaling is a key feature of FXS, leading to impairments in executive functioning (Hoeft et al., 2007), which is in line with the altered white matter connectivity in fronto-striatal connections, described above.

The **neurofibromin 1** gene (*NF1*) located on chromosome 17q11.2 codes for neurofibromin, a protein which is thought to be a regulator of the RAS signal transduction pathway and necessary for embryonic development. Neurofibromatosis type 1 (NF1) is caused by mutations in the gene, often leading to the synthesis of truncated or otherwise non-functional proteins. We found 14 studies investigating effects of *NF1* on brain structure and four investigating brain function. Additional studies of brain structural and functional connectivity have been conducted. While most studies included children and adolescents, a few studies have included adults as well (Duarte et al., 2014; Karlsgodt et al., 2012; Pride et al., 2014; Violante et al., 2012; Wignall et al., 2010; Zamboni et al., 2007) (**Table 7**).

The structural brain abnormalities most commonly seen in subjects with NF1 are T2 hyperintensities and an increased brain volume. T2 hyperintensities are areas of high signal intensity on T2-weighted MR images also referred to as 'unidentified bright objects' (UBOs). Although their association with cognitive and intellectual deficits remains controversial, thalamic hyperintensities have repeatedly been associated with cognitive impairments (Payne et al., 2010). Multiple studies have investigated the characteristics of UBOs. UBOs are found in almost all children with NF1, but reports on whether their volume and number increases or decreases with age are inconsistent (Gill et al., 2006; Griffiths et al., 1999; Kraut et al., 2004). A few studies have used diffusion tensor imaging (DTI) to characterize white matter microstructure and integrity of UBOs by measuring the degree and directionality of diffusivity. Higher apparent diffusion coefficient (ADC) and (radial) diffusivity values and lower fractional anisotropy (FA) values have been found in UBOs compared to normal appearing white matter (Ertan et al., 2014; van Engelen et al., 2008). These findings can be explained by myelin deficiency and axonal damage. An increase in brain volume is observed in children with NF1, which was found to be due to increases in white matter volume (Said et al., 1996; Steen et al., 2001), gray matter volume (with an increased gray to white matter ratio especially in younger subjects (Moore et al., 2000)), or both gray and white matter volume (Karlsgodt et al., 2012). These volume increases involve temporal, parietal, occipital, and frontal regions (Duarte et al., 2014; Greenwood et al., 2005; Pride et al., 2014). In addition, the corpus callosum seems larger in cases compared to controls, which has been found in

children with NF1 as well as adults, marking it as a robust finding for NF1 (Duarte et al., 2014; Moore et al., 2000; Violante et al., 2013; Wignall et al., 2010). In addition to the investigation of UBOs, DTI studies have been used to study microstructural integrity in NF1 more broadly. Increased ADC values (Ertan et al., 2014; Nicita et al., 2014; van Engelen et al., 2008) and decreased FA values (Ertan et al., 2014; Ferraz-Filho et al., 2012) are found widespread across the brain. Karlsgodt et al. also found increased radial diffusion, which may be explained by decreased myelination or axonal packing density (2012). Differences in radial diffusivity have also been observed at the genu and anterior body of the corpus callosum (Wignall et al., 2010). The change in corpus callosum size and connectivity observed in NF1 may have functional importance, as they have been associated with academic achievement and visual-spatial and motor skills (Moore et al., 2000).

Three fMRI studies have investigated visual-spatial processing in subjects with NF1, and one study investigated phonologic processing (**Table 7**). During visual-spatial processing, decreased activation in the primary visual cortex was found for individuals with NF1 compared to controls (Clements-Stephens et al., 2008), although an earlier study reported contrasting findings of increased posterior (occipital) cortex activation relative to lateral/inferior frontal activation (Billingsley et al., 2004). A later study did confirm that both children and adults with NF1 showed deficient activation of the low-level visual cortex during tasks specifically designed to activate magnocellular and parvocellular pathways (Violante et al., 2012). During such magnocellular-biased stimulation, NF1 patients did not deactivate regions belonging to the brain default-mode network as would be expected during cognitively demanding tasks (Violante et al., 2012).

The tumor growth suppressor genes **tuberous sclerosis 1** (*TSC1*) and **tuberous sclerosis 2** (*TSC2*) code for the hamartin and tuberin proteins, respectively. Mutations in either *TSC1* or *TSC2* disrupt the function of the GTPase-activating protein (GAP) complex formed by these proteins that regulates mTOR signaling. The neurocutaneous syndrome tuberous sclerosis complex (TSC), characterized by benign hamartomas in multiple organ systems, is caused primarily by these mutations. In the brain, the hamartomas manifest as subendymal giant cell astrocytomas, subendymal nodules (SEN), and tubers. Tubers show disrupted cortical architecture and contain a number of atypical cells. For TSC, structural MRI and DTI studies have been conducted investigating both typical neuropathological lesions, especially tubers, and normal-appearing brain matter (**Table 7**). A consistent imaging determinant of the cognitive phenotype in TSC has not been established. Findings of an inverse correlation of tuber number and cognitive functioning have not been consistent (Ridler et al., 2004). Tuber/brain proportion may be a better predictor of IQ than tuber load, although the age of seizure onset in patients seemed to predict cognitive functioning best (Jansen et al., 2008b). However, abnormal brain structure and connectivity unrelated to tubers are likely also important factors contributing to the neurobehavioral abnormalities in TSC. Decreased white matter volume of major intrahemispheric tracts has been found in

adults with TSC compared to age-matched controls, as has a decrease of gray matter volume in several cortical and subcortical structures (Ridler et al., 2001; Ridler et al., 2007). Reduced volume in the cerebellum has been associated with tuber-associated loss of the underlying parenchyma (Jurkiewicz et al., 2006; Marti-Bonmati et al., 2000). Reduced cerebellar volume was observed in all cerebellar regions in a more recent study, with strongest volume reductions in patients with a mutation in *TSC2* (Weisenfeld et al., 2013). The finding of reduced cerebellar volume is in line with mouse models showing cerebellar involvement in TSC (Reith et al., 2011). White matter abnormalities are another typical finding in TSC. DTI studies generally report increased ADC values and decreased FA values in individuals with TSC compared to controls, in tubers and white matter lesions, but also in other white matter portions (**Table 7**). Compared to contralateral white matter or white matter in control subjects, increased ADC values were found in cortical tubers, and higher ADC and lower FA values were found in white matter lesions (Piao et al., 2009). A recent study also found increased radial diffusivity values and decreased FA values in cortical tubers and white matter lesions (Dogan et al., 2015). Hypomyelination, gliosis, and heterotopic cells may lead to ADC and FA changes observed in such lesions (Alexander et al., 2007). Abnormalities have also been reported in normal-appearing white matter in individuals with TSC compared to control groups. Decreased FA and increased ADC, especially in corpus callosum and internal and external capsules, have been reported repeatedly (Krishnan et al., 2010; Peters et al., 2012; Simao et al., 2010). A recent whole-brain analysis of white matter connectivity showed that increased radial diffusivity exists throughout the brains of TSC patients and that interhemispheric connectivity is decreased (Im et al., 2015).

The **methyl CpG binding protein 2** gene (*MECP2*) is located on the short arm of chromosome X (Xq28) and codes for the protein MECP2. MECP2 acts as a modifier of gene expression and is highly expressed in the brain. Mutations in *MECP2* are the cause of Rett syndrome, a disorder primarily affecting female patients. Brain weight is reduced in Rett syndrome, particularly that of cerebral hemispheres. Although the anatomical basis for this reduction is not completely clear, it has been suggested that it is caused by defective neuronal maturation for which MECP2 is essential, rather than by atrophy (Armstrong, 2005). Only few imaging studies have been conducted in series of patients with Rett syndrome (**Table 7**). All investigated brain structure in girls. These studies confirmed a wide-spread reduction in cerebral white and gray matter volumes, the latter most pronounced in subcortical nuclei including the caudate nucleus and in prefrontal, posterior-frontal, and anterior-temporal (Reiss et al., 1993; Subramaniam et al., 1997) and parietal regions (Carter et al., 2008). Using DTI, evidence of reduced white matter integrity was found in frontal regions, corpus callosum, and internal capsule. FA was also reduced in the superior longitudinal fasciculus, but only in patients who had little or no ability to speak (Mahmood et al., 2010).

Table 7: Imaging genetics studies in intellectual disability syndromes (fragile X syndrome (*FMR1*), tuberous sclerosis (*TSC1* and *TSC2*), neurofibromatosis type 1 (*NF1*), and Rett syndrome (*MECP2*)). No studies for Timothy syndrome (*CACNA1C*) were retrieved in our search of the literature.

Disorder	Gene	Imaging modality	Imaging phenotype	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
Fragile X syndrome	*FMR1* full mutation	sMRI	Quantitative morphometry	Subgroups of 51 FXS; 120 HC	↑ CN volume, and lateral ventricle (in males).	(Reiss et al., 1995)
			Hippocampus and amygdala volume	10 FXS (5 ‡ (6.3); 5 † (9.0)); 10 HC (5 ‡ (6.4); 5 † (8.7))	↑ Right hippocampal volume.	(Kates et al., 1997)
			Regional brain volumes	10 FXS (9.0); 10 HC (8.5)	↑ CN and ventricular volumes.	(Kaplan et al., 1997)
			Tissue volumes	10 FXS (9.1); 10 HC (8.5)	↑ CN GM volume.	(Reiss et al., 1998)
			TBM	36 FXS (14.66); 33 HC (14.67)	↑ CN and lateral ventricle volumes, and trend-level parietal and temporal WM ↑.	(Lee et al., 2007)
			GM VBM and manual tracing; multivariate pattern classification	51 FXS (35 months); 32 HC (29.7 months); 18 DD (34.8 months) ‡	↓ GM volumes in regions including hypothalamus, insula, medial and lateral PFC. Spatial patterns that discriminated FXS from other groups included a medial to lateral gradient of increased and decreased regional brain volumes in posterior vermis, amygdala, and hippocampus.	(Hoeft et al., 2008)
			CN, hippocampus, putamen, amygdala volume	52 FXS (2.9); 63 ASD (2.8); 19 DD (3,0); 31 HC (2.6) ‡	↑ CN volume compared to all control groups. FXS: ↓ amygdala volume. ASD:↑ amygdala volume.	(Hazlett et al., 2009)
			VBM of regional GM	10 FXS (28.9); 10 ASD (30.1); 10 HC (29.4)	FXS: ↑ GM volumes within frontal, parietal, temporal, and cingulate gyri, and in CN and cerebellum compared to ASD. FXS: ↑ GM volumes in frontal gyri and CN and ↓ GM volumes in cerebellar, parietal and temporal regions compared to HC. ASD: ↑ GM volumes in frontal and temporal gyri compared to FXS and ↓ GM cerebellar volumes compared to HC.	(Wilson et al., 2009)

Table 7: Continued.

Disorder	Gene	Imaging modality	Imaging phenotype	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
			Total and regional insular volumes	11 FXS (5 † (15.3); 6 ‡ (16.3)); 8 HC ((5 † (16.5); 3 ‡ (13.3)); 11 DD (6 † (16.4); 5 ‡ (16.0))	↓ Total, anterior and posterior insular volumes compared to HC and DD.	(Cohen et al., 2011)
			Univariate VBM; multivariate pattern classification and clustering.	52 FXS (2.90); 63 ASD (2.77); 31 HC (2.55); 19 DD (2.96) ‡	↓ (for FXS) and ↑ (for ASD) volumes of frontal and temporal GM and WM regions (including medial PFC, OFC, superior temporal region, temporal pole, amygdala, insula, and dorsal cingulum) compared to HC. Overall pattern of brain structure in ASD resembles that of HC more than FXS.	(Hoeft et al., 2011)
			Regional brain bulk volumes (stereology) and GM and WM volume (VBM)	17 FXS (30); 18 HC (35) ‡	↑ CN, parietal lobes and right brainstem bulk volume. ↓ Left frontal lobe volume. ↑ GM volumes of fronto-striatal regions including CN. ↑ WM in regions extending from brainstem to para-hippocampal gyrus, and from left cingulate cortex to CC.	(Hallahan et al., 2011)
			Age-related change in regional brain volumes	59 FXS (36 † (16.0); 23 ‡ (15.2)) (19 with longitudinal data); 83 HC (47 † (15.8); 36 ‡ (15.5)) (17 with longitudinal data)	Consistent FXS related volume differences in CN compared to HC across adolescence. Aberrant maturation of PFC gyri.	(Bray et al., 2011)
			Cortical volume, thickness, complexity, surface area and gyrification index	11 FXS (9.16) (6 FXS; 5 FXS+ASD); 10 HC (8.25) ‡	FXS: ↑Cortical volume, thickness and complexity compared to HC. FXS+ASD: ↑ Left parietal lobe volume, ↓ gyrification specifically in the left temporal and a trend for ↓ right frontal surface area compared to FXS.	(Meguid et al., 2012)

Table 7: Continued.

Disorder	Gene	Imaging modality	Imaging phenotype	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
			Total brain, regional (lobar) and subcortical volumes; brain growth	53 FXS (2.9); 68 ASD (2.8); 19 DD (3.0); 31 HC (2.6) ‡	FXS: ↑ Global brain volumes compared to HC but not ASD. ↑ Temporal lobe WM, cerebellar GM, and CN volume compared to ASD. ↓ Amygdala volume compared to ASD. Rate of brain growth from 2 to 5 years similar to HC.	(Hazlett et al., 2012)
			Relationship repetitive behaviors and CN volume	41 FXS (4.6) (16 FXS+ASD (4.8)); 30 ASD (4.7) ‡	FXS: Positive correlation of self-injury with CN volume. ASD: Positive correlation of compulsive behaviors with CN volume.	(Wolff et al., 2013)
			CN volume and topography	48 FXS (21.3); 28 IQ-matched controls (19.5); 36 HC (19.7)	↑ CN compared to both control groups, with ↑ bilateral CN radial distance, ↑ dorsolateral CN head and ventromedial CN body radial distances.	(Peng et al., 2014)
			CN and hippocampal volume	14 FXS+ASD (22.6); 17 HI (22.0); 25 HC (21.6) ‡	FXS: ↑ Hippocampus and CN volume compared to HC. HI: ↓ Hippocampal volumes.	(Molnar and Keri, 2014)
		DTI	Whole-brain, frontal-caudate, and sensory-motor tract FA	10 FXS (16.7); 10 HC (17.1) †	↓ FA in WM in fronto-striatal pathways and parietal sensory-motor tracts.	(Barnea-Goraly et al., 2003)
			Ventral frontostriatal WM	17 FXS (2.8); 13 HC (2.3); 8 DD (3.0) ‡	↑ Density of fibers localized in left ventral frontostriatal pathway.	(Haas et al., 2009)
			Voxel-based comparison of anisotropy and diffusivity	18 FXS (11.01); 25 22q11.2DS (10.75); 17 TS (10.56); 41 HC (10.6) †	FXS: ↓ FA in posterior limbs of internal capsule, posterior thalami, and precentral gyrus.	(Villalon-Reina et al., 2013)

Table 7: Continued.

Disorder	Gene	Imaging modality	Imaging phenotype	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
		sMRI	GM density (VBM)	17 FXS (17.5); 16 HC (16.3)	↑ GM density in bilateral caudate head, left hippocampus, left planum temporale, left angular gyrus, and left superior parietal lobule.	(Hall et al., 2013)
		rs-fMRI	Fractional Amplitude (fALFF); functional connectivity (group ICA and dual regression)		↓ GM density in bilateral insular cortex, precuneus cortex, thalamus, and subgenual cingulate cortex. ↓ Functional connectivity in salience, precuneus, left executive control, language, and visuospatial networks. ↓fALFF in bilateral insular, precuneus, and ACC.	
		fMRI	ROI activation during 1-back and 2-back visuospatial working memory tasks	10 FXS; 15 HC †	No change in activation between 1-back and 2-back tasks in IFG, middle frontal gyrus, superior parietal lobule, and supramarginal gyrus, while HCs showed ↑ activation.	(Kwon et al., 2001)
			Activation during a counting Stroop task	14 FXS; 14 HC † (15.4) †	↓ Activation in orbitofrontal gyrus, insular cortex, superior temporal gyrus. No activation in inferior/superior parietal lobe as seen in HC.	(Tamm et al., 2002)
			FG and STS activation in response to face and gaze stimuli	11 FXS (16.4); 11 HC (15.5) †	↓ Left STS activation to all stimuli. No greater FG activation to forward faces compared to angled faces as seen in HC.	(Garrett et al., 2004)
			Go/nogo task	10 FXS (15.4); 10 DD (14.6); 10 HC (16.7) ‡	↓ Activation in right ventrolateral PFC and right caudate head, and ↑ left ventrolateral PFC activation compared with both control groups. Positive correlation between task performance and activation in left ventrolateral PFC.	(Hoeft et al., 2007)

Table 7: Continued.

Disorder	Gene	Imaging modality	Imaging phenotype	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
			Emotional attribution task	10 FXS (16.4); 10 HC (15.6) †	↓ ACC activation for neutral compared to scrambled faces. ↓ CN activation for sad compared to scrambled faces. FXS: ↑ Negative correlation between IQ and insula activation for neutral compared to scrambled faces. HC: ↑ Positive correlation between IQ and ACC activation for neutral compared to scrambled faces.	(Hagan et al., 2008)
			Activation during face encoding	11 FXS (18.5); 11 HC (18.7)	↓ Activation of prefrontal regions including medial and superior frontal cortex during successful face encoding. Negative correlation social anxiety and brain activity during face encoding.	(Holsen et al., 2008)
			Whole-brain and ROI activation during directed or averted eye gaze stimuli	13 FXS (15.5); 10 DD (16.1); 13 HC (15.0) ‡	↓ PFC activation and ↑ left insula activation to direct eye gaze stimuli. ↑ Sensitization in left amygdala with successive exposure to direct gaze compared to controls.	(Watson et al., 2008)
			Auditory temporal discrimination task	10 FXS (18.7); 10 HC (14.7) †	↑ Activation in a left-lateralized network including left medial frontal gyrus, left superior and middle temporal gyrus, left cerebellum, and left brainstem (pons).	(Hall et al., 2009)
			Brain activity during a gaze habituation task	30 FXS (20.9); 25 HC (19.0)	↓ Neural habituation and significant sensitization in cingulated gyrus, fusiform gyrus and frontal cortex in response to gaze stimuli.	(Bruno et al., 2014)
Neuro-fibromatosis type 1	*NF1*	sMRI	Cerebral GM and WM	22 NF1; 20 HC	↑ Brain volume, especially WM.	(Said et al., 1996)
			Number, volume, distribution and change in time of UBOs	46 NF1 (7.8) (28‡; 18†)	UBOs found in 93% of subjects, localized most commonly in GP (30.4%), cerebellum (23.5%), and midbrain (16.2%). ↑ UBO number and volume between 4 to 10 years with a reduction in subjects aged 10+ years.	(Griffiths et al., 1999)

Table 7: Continued.

Disorder	Gene	Imaging modality	Imaging phenotype	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
			24 ventricular and parenchymal dimensions and area calculations	27 NF1 (8.8) (20‡; 7†); 43 HC (5.9) (22‡;21†)	↑ Bicaudate width, biatrial width, and biparietal diameter, but not hemispheric length. ↑ Iter measures, descending sigmoid sinus, and ↑ brainstem height (age-specific).	(DiMario et al., 1999)
			TBV, GM, WM, CSF, CC regions and hyperintensities	52 NF1 (10.9); 19 HC (9.8)	↑ TBV due to ↑ GM volume. ↑ CC size. ↑ Group differences in GM to WM ratio in younger compared to older subjects.	(Moore et al., 2000)
			Morphometric and volumetric measures of (midline) structures; GM and WM volume	18 NF1 (range 6.2-14.7); 60 HC (range 4.5-16.1)	↑ Bilateral hyperintensities and ↑ midline structure size in macrocephalic compared to normocephalic NF1. ↑ Brain volume and WM volume but not GM or ventricular volume in macrocephalic subjects compared to HC.	(Steen et al., 2001)
			Surface area, GM volume, and asymmetry of the PT and PP	24 NF1 (11.1); 24HC (11.8)	↓ Left PT surface area and GM volume and ↑ symmetry between left and right PT in NF1 boys compared to NF1 girls and HC.	(Billingsley et al., 2002)
			Number of affected regions, UBO volume and number	12 NF1 (13.0)	UBO prevalent in GP/internal capsule. ↓UBO locations, number and/or volume for all regions except cerebellar hemispheres between ages 7 to 12 years and ↑ during adolescence.	(Kraut et al., 2004)
			GM and WM volumes	36 NF1 (9.3); 39 HC (9.5)	↑ GM volumes predominantly in temporal, parietal and occipital regions and WM volumes predominantly in frontal lobe.	(Greenwood et al., 2005)
			Frequency, signal characteristics and localization of T2 hyperintensities at different ages	103 NF1 (13.9)	↓ Frequency, size, and intensity of T2 hyperintensities in BG and cerebellum/brainstem with age. No differences in hemispheric lesions with age.	(Gill et al., 2006)

Table 7: Continued.

Disorder	Gene	Imaging modality	Imaging phenotype	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
			Regional subcortical volumes; cortical volume, thickness, surface area and gyrification	14 NF1 (11.3); 14 HC (11.9)	↑ Volume of thalami, right CN and middle CC. ↓ Gyrification indices in frontal and temporal lobes, insula, cingulate cortex, parietal and occipital regions. No differences in cortical volume, thickness and surface area.	(Violante et al., 2013)
			SVM; VBM	21 NF1 (11.1); 29 HC; 18 NF1 (33.1); 31 HC (35.0)	SVM classifiers correctly classified 94% of cases (sensitivity 92%; specificity 96%).	(Duarte et al., 2014)
			GM and WM volume	16 NF1 (29.8); 16 HC (33.1)	↓ GM volume of superior frontal gyrus, orbital gyrus and right STG ↑ GM volume in frontal, temporal, ,parietal and limbic lobes	(Pride et al., 2014)
		sMRI	TBV; CC morphology	10 NF1 (range 20-68): 10 HC (range 21-64)	No differences in TBV. ↑ CC length (10%), CC area (20%).	(Wignall et al., 2010)
		DTI	CC diffusion characteristics		↑ Minor eigenvalues at genu of CC.	
			GM and WM volume	14 NF1 (24); 12 HC (22.7)	↑ GM and WM volume.	(Karlsgodt et al., 2012)
			TBSS		↓ FA and radial diffusion and ↑ ADC with greatest magnitude in frontal lobe.	
		DTI	FA and ADC brainstem, basal ganglia, thalamus, CC, and frontal and parietooccipital WM regions	10 NF1 (25.8); 10 HC (26.3)	↑ ADC and ↓ FA in all regions of interest.	(Zamboni et al., 2007)
			Diffusion characteristics (ADC, FA, A(m), eigenvalues) healthy and disordered brain matter	50 NF1 ((21 female (12.2); 29 male (12.3)); 8 HC	↑ ADC and eigenvalues in UBO compared to normal-appearing sites ↑ ADC in normal-appearing sites compared to HC. No differences in FA or A(m) in most regions.	(van Engelen et al., 2008)
			FA BG, cerebellum, pons, thalamus	44 NF1(12.8); 20 HC (14.1)	↓ Bilateral cerebellar and thalamic FA.	(Ferraz-Filho et al., 2012)

Table 7: Continued.

Disorder	Gene	Imaging modality	Imaging phenotype	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
			FA, ADC CN, putamen, GP, thalamus	14 NF1 (16.3) (8 with UBOs; 6 without UBOs and 9 <18 years;5 > 18 years); 8 HC (16.1)	↑ ADC in CN, putamen, GP, thalamus.	(Nicita et al., 2014)
			ADC,FA, RD, eigenvalues for 7 GM and 8WM ROIs; WM trajectories for adjacent WM tracts of NBOs	14 NF1 (7.2); HC	↑ ADC and eigenvalues in GM and WM UBOs compared to contralateral normal-appearing sites and HC and ↓ FA compared to HC. Three out of 18 UBOs disrupt WM tracts. ↑ADC, lambda(2) and radial diffusivity of WM UBOs in patients with neurological symptoms compared to patients without.	(Ertan et al., 2014)
		fMRI	Activity in ten ROIs during phonologic processing	15 NF1 (14.4); 15 HC (15.3)	Inferior and dorsolateral PFC activation relative to posterior activation ↑ during auditory phonologic processing and ↓ during orthographic processing.	(Billingsley et al., 2003)
			Occipital and parietal cortex activity during visual-spatial processing	15 NF1 (14.4); 15 HC (15.3)	↑ Posterior cortex activation relative to lateral and inferior frontal activation.	(Billingsley et al., 2004)
			Activation in frontal, temporal, parietal, and occipital regions during visuospatial processing	13 NF1 (9.8); 13 HC (9.8)	↑ Left instead of right hemisphere activation. ↓ Activation in primary visual cortex.	(Clements-Stephens et al., 2008)
			Early cortical visual pathway and DN activation during visual stimuli activating magnocellular and parvocellular pathways	15 NF1 (11.7); 24 HC (12.0); 13 NF1 (33.1)†; 15 HC (32.7)†	↓ Activation of low-level visual cortex. ↓ Deactivation or ↑ activation of midline regions of DN during magnocellular- biased stimulation.	(Violante et al., 2012)

Table 7: Continued.

Disorder	Gene	Imaging modality	Imaging phenotype	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
		rs-fMRI	Ventral ACC, amygdala, OFC, PCC RSFC	14 NF1 (12.5); 30 HC (12.3)	↑ Connectivity between: left ventral ACC and frontal cortex, insula, and subcortical areas (CN, putamen); left amygdala and frontal cortex, insula, supramarginal gyrus, and PCC/precuneus; left OFC and frontal and subcortical areas (CN, pallidum).	(Loitfelder et al., 2015)
Tuberous sclerosis complex	*TSC1/TSC2*	sMRI	Number and location of cerebellar tubers and volumes of underlying parenchyma	34 TSC (8.9)	Mean tuber number was 14.3 and 44.1% of subjects showed both cerebral and cerebellar tubers and had more global cortical lesions than subjects with cerebral tubers only. ↓ Focal volume associated with tubers in cerebellum.	(Marti-Bonmati et al., 2000)
			GM, WM and CSF volume	10 TSC (41.5); 8 HC (40.0)	↓ GM volume in medial temporal lobes, posterior cingulate gyrus, thalamus, BG and right fronto-parietal cortex. ↓ Of limbic and subcortical GM volume negatively correlated with tuber count. ↓ WM of longitudinal fasciculi and other major intrahemispheric tracts. ↑ Cerebellar WM.	(Ridler et al., 2001)
			Tuber distribution and lesion load	25 TSC (39.0)	Highest tuber frequency in frontal lobes and highest tuber density in parietal regions with variation in tuber density but no lateralization of tubers. Nodules were located predominantly in CN. Tuber and nodule volumes positively correlated. ↑ Tuber volume in subjects with a history of epilepsy.	(Ridler et al., 2004)
			Characteristics of cerebellar lesions	73 TSC (range 0-28 years)	16.4% of TSC subjects showed cerebellar lesions. Six subjects showed atrophy of cerebellar parenchyma around tubers.	(Jurkiewicz et al., 2006)
			GM, WM and CSF volume and lesion load	25 TSC (39.3); 25 HC (34.3)	↓ Subcortical GM volume in regions including thalamus, BG, insula, and cerebellum. ↓ WM in intrahemispheric tracts.	(Ridler et al., 2007)
			Tuber number and tuber/brain proportion	58 TSC (20.6) (19 *TSC1* (25.0); 34 *TSC2* (19.0))	↑ Tubers and tuber/brain proportion in *TSC2* compared to *TSC1* subjects and in subjects with a mutation deleting or directly inactivating tuberin GAP domain compared to subjects with an intact GAP domain.	(Jansen et al., 2008a)

Table 7: Continued.

Disorder	Gene	Imaging modality	Imaging phenotype	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
			Tuber number and tuber/ brain proportion as determinants of seizures and cognitive function	61 TSC (17.9) (14 *TSC1*; 30 *TSC2*)	Tuber/brain proportion was inversely related to age at seizure onset and intelligence.	(Jansen et al., 2008b)
			Presence of SENs and SGCTs	81 TSC (28)	15% of TSC subjects showed SGCTs. 62% showed SENs, 24% of which also showed SGCTs. ↑ SGCT volume at follow-up.	(Michelozzi et al., 2013)
			Cerebellar volume	36 TSC (9.7)(19 *TSC2*; 7 *TSC1*); HC (9.7)	↓ Cerebellar volume, with strongest effect in subjects with *TSC2* mutations.	(Weisenfeld et al., 2013)
			(Cyst-like) tuber/ brain proportion and tuber number in relation to age at seizure onset	23 TSC (12.4)	Tuber/brain proportion and number of tubers, but not cyst-like tuber/brain proportion and number of cyst-like tubers, were negatively correlated with age at seizure onset.	(Nakata et al., 2013)
		DTI	ADC, FA of epileptogenic tubers	15 TSC	↑ ADC values in subtuber WM in epileptogenic tubers compared to nonepileptogenic tubers.	(Chandra et al., 2006)
			ADC of NAWM in frontal, parietal and occipital lobes, and pons	23 TSC (12); 18 HC	↑ ADC values in frontal WM and pons for age group between 96 and 144 months and in right parietal and occipital WM for subjects older than 144 months.	(Arulrajah et al., 2009)
			ADC, FA in tubers and WM lesions	14 TSC (15.1)	↑ ADC values in cortical tubers. ↑ ADC values and ↓ FA values in WM lesions compared with contralateral regions.	(Piao et al., 2009)
			FA, diffusion characteristics in ROIs in or adjacent to cortical tubers in epileptogenic and non-epileptogenic zones	12 TSC (8.2)	↓ FA of cortical tubers in epileptogenic compared to non-epileptogenic zones. ↑ Radial diffusivity and ↓ FA in NAWM in epileptogenic zones compared to non-epileptogenic zones.	(Widjaja et al., 2010)

Table 7: Continued.

Disorder	Gene	Imaging modality	Imaging phenotype	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
			FA, trace, eigenvalues CC and internal capsules, in relation to tuber load	12 TSC (9.2); 23 HC (11.1)	Tubers were found in frontal lobes (144), parietal lobes (64), temporal lobes (42), occipital lobes (57) and insular cortex (7). ↓ FA, ↑ trace and average lambda(3) in CC and ↑ trace in internal capsules. Tuber volume correlated with multiple DTI characteristics in CC and internal capsules.	(Simao et al., 2010)
			Diffusion characteristics geniculocalcarine tract, internal capsule, temporal gyri and splenium of the CC	10 TSC (range 1.5-25 years); 6 HC (range 1.1-25 years)	↓ FA in geniculocalcarine tracts and splenium of CC. ↓ Axial diffusivity in internal capsule, STG, and geniculocalcarine tracts. ↑ Mean and radial diffusivity in splenium of CC.	(Krishnan et al., 2010)
			FA, mean radial and axial diffusivities of CC	40 TSC (7.2) (12 with ASD); 29 HC (7.7)	↓ Average FA and ↑ diffusivity values in CC. ↓ Average FA in TSC +ASD subjects compared to HC and TSC -ASD subjects (who showed no differences).	(Peters et al., 2012)
			Diffusion characteristics in major tracts	16 TSC (13.0); 12 HC (15.3)	↓ FA and axial diffusivity in wide-spread WM regions. ↓ Number of fibers and number of tract points of commissural fibers, projection fibers and major WM tracts.	(Wong et al., 2013a)
			Diffusion characteristics of RMLs, tubers, SENs, cerebellar lesions and SGCT and NAWM	30 TSC (15.5); 16 HC (7 children (9); 9 adults (36))	Mean of 47 RMLs, 27 tubers, and 10 SENs per TSC subject. Inverse correlation of RML FA and MD. No differences NAWM FA and MD.	(van Eeghen et al., 2013)
			FA dorsal language circuit tract	38 TSC (10 TSC + ASD; 17 TSC - ASD); 24 HC	↓ FA values in dorsal language circuit tract. ↓ FA in WM close to Geschwind's territory and WM close to Broca's area in TSC +ASD compared to TSC -ASD subjects.	(Taquet et al., 2014)
			FA, ADC, axial and radial diffusivity of tubers and WM lesions	18 TSC (9.3)	↓ FA and ↑ ADC and axial and radial diffusivity values in tubers compared to contralateral normal regions. ↑ Radial diffusivity and ↓ FA in WM lesions.	(Dogan et al., 2015)

Table 7: Continued.

Disorder	Gene	Imaging modality	Imaging phenotype	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
			Global and regional WM connectivity	20 TSC (range 3-24 years)(11 TSC+ DD; 9 TSC - DD; 20 HC (range 2-23 years)	↓ Interhemispheric connectivity. ↑ MD, positively correlated with tuber load severity. ↑ MD in TSC + DD subjects compared to TSC – DD subjects.	(Im et al., 2015)
Rett syndrome	*MECP*	sMRI	TBV, cortical GM and WM, subcortical gray nuclei, CSF volumes	11 RTT (10.1); 15 HC (11.2) †	↓ Cerebral volume ↑ Loss of GM in comparison to WM, with largest decrease in frontal regions and CN and midbrain volume.	(Reiss et al., 1993)
			TBV, cortical GM and WM, subcortical GM, CSF and posterior fossa volumes	20 RTT (9.8); 20 HC (9.0) †	↓ GM volume most pronounced in prefrontal, posterior-frontal, and anterior-temporal regions. ↓ WM volume uniformly throughout brain. ↓ CN volume. No differences in midbrain volumes.	(Subramaniam et al., 1997)
			Absolute and relative changes in GM and WM volumes	23 RTT (8.6) (12 more severe (8.8); 10 less severe (8.3)); 25 HC (8.9)†	↓ Absolute volume throughout the brain ↓ Relative parietal lobe GM volume, particularly dorsal. ↓ Cortical WM volume. ↓ Anterior frontal lobe volumes in more severely affected subjects.	(Carter et al., 2008)
		DTI	Regional FA	32 RTT (5.5); 37 HC (6.1)†	↓ FA in genu and splenium of CC and external capsule, and regions of cingulate, internal capsule, posterior thalamic radiation, and frontal WM. No differences in visual pathways. ↓ FA in superior longitudinal fasciculus in patients who were nonverbal or speaking only single words.	(Mahmood et al., 2010)

22q11.2DS= 22.q11.2 deletion syndrome, ACC= anterior cingulate cortex, ADC= apparent diffusion coefficient, A(m)= axial anisotropy, ASD= autism spectrum disorder, BG=basal gangli, CC= corpus callosum, CN= caudate nucleus, CSF= cerebrospinal fluid, DD= developmental delay, DN= default network, DTI = diffusion tensor imaging, FA= fractional anisotropy, fALFF = fractional amplitude of low-frequency fluctuations, FG= fusiform gyrus, fMRI= functional MRI, FXS= fragile X syndrome, GAP= GTPase activating protein, GM= grey matter, GP= globus pallidum, HI= hypoxic injury, IFG= inferior frontal gyrus, IPL= inferior parietal lobule, IPS= intraparietal sulcus, MCP= middle cerebellar peduncle, MD= mean diffusivity, MTI=magnetization transfer imaging, NAWM= normal-appearing white matter, NF1= neurofibromatosis 1, OFC= orbitofrontal cortex, PCC= posterior cingulate cortex, PFC= prefrontal cortex, PP= planum parietale, PT= planum temporale, RML= radial migration lines, ROI= region of interest, RSFC= resting state functional connectivity; RTT= Rett syndrome, SCP= superior cerebellar peduncle, SEN= subependymal nodule, SGCT= subependymal giant cell tumour, sMRI= structural MRI, STG= superior temporal gyrus, STS= superior temporal sulcus, SVM= support vector machines, SWM= spatial working memory, T1= timepoint 1, T2= timepoint 2, TBM = tensor-based morphometry, TBV= total brain volume, TPJ= temporoparietal junction, TS= turner syndrome, TSC= tuberous sclerosis, TWM = temporal working memory, UBO = unidentified bright objects, VBM= voxel based morphometry, WM= white matter, † female, ‡ male.

Discussion

In this review, we set out to summarize the literature on imaging genetics studies in neurodevelopmental disorders. This being a very broad field, we focused on three most frequent and often comorbid disorder spectra, ADHD, ASDs, and selected forms of ID, and we only considered MRI-based imaging genetics studies. Further restriction of the search space was achieved by focusing on genes harboring common genetic variants with the most consistent evidence for association with ADHD and ASDs, and by selecting five relatively common ID disorders with frequent ADHD/ASDs comorbidity implicating single genes. The review was driven by the wish to learn more about the mechanisms by which genetic factors influence disease-related behavior specific to the individual disorders and their clinical overlap.

At the level of the individual genes, the most extensively studied candidate gene is the *SLC6A4* (*5HTT*) gene encoding the serotonin transporter (associated with both ADHD and ASDs). Limitations regarding power of individual studies and hypothesis-driven designs aside, the fMRI-based imaging genetics literature on this gene does show a remarkably coherent picture of functional genetic variation leading to hyperactivation of the amygdala and connected areas in conjunction with functional dysconnectivity amongst those areas. However, since much of this research has been performed in healthy individuals only, the link to cognition in ADHD and ASD patients needs further investigation. Findings for *SLC6A3* (*DAT1*) and *DRD4*, which have also been studied quite often already, still lack the consistency observed for *SLC6A4* (*5HTT*), partly due to the much less restricted focus on a particular cognitive domain, and thus more 'patchy' literature.

The most consistent findings observed in all of the imaging genetics literature reviewed here are for the different genetic variants for ID. This is likely linked to the severity of the variants present in the patients, with those for ID being rare and most damaging. Consistent are finding for increased caudate volume and reduced cerebellum due to *FMR1* mutations, and for T2 hyperintensities and increased brain volume in patients carrying *NF1* mutations. However, in terms of finding overlap between different forms of ID, we find that conclusiveness of studies still is limited, as most concentrated on a limited set of (often non-overlapping) features. Tubers and T2 hyperintensities have received a lot of attention in studies of TSC and NF1, for example, although reports on their contribution to cognitive deficits are inconsistent. In recent years, DTI studies have produced evidence that tissue microstructure and white matter connectivity patterns are affected in all ID disorders, and often in widespread brain areas. Effects on brain volumes are also often widespread, but can go in opposite directions, with reductions in total brain volume in Rett, but increases in NF1. One may conclude that while altered (structural) connectivity is likely to play a role in ID etiology, MRI at its current resolution (1.5 – 4 Tesla), does not allow a sufficiently detailed

view on the brain to understand the neuroanatomical overlap between disorders (Williams and Casanova, 2011).

Similar to the situation amongst the ID disorders, there seems to be little overlap between the findings for different genes in ADHD or ASD. This is likely to be heavily influenced by the strong focus on regions and cognitive domains of interest (consistent with the limited power of many of the studies published to date). Some overlap is seen, e.g., for *DAT1* and *DRD2*, both of which have been studied for their effects on striatal phenotypes. (Appropriately powered) brain-wide studies and phenome-wide association study (PheWAS)/RDoc-like approaches (Cuthbert and Insel, 2013; Pendergrass et al., 2011) would help to determine, whether the apparent specificity of brain phenotypes for individual genes is real. An important observation is that gene expression does not predict/limit the location of effects of a genetic factor (e.g., *SLC6A3/DAT1* shows effects outside of its region of gene expression), most likely through effects on structural and/or functional connectivity.

Did the reported imaging genetics findings help us understand the comorbidity between different neurodevelopmental disorders? This would be expected, since several of the genes implicated in ID, ASD, and ADHD function in the same or overlapping molecular networks (Poelmans et al., 2011; Rudie et al., 2012; van Bokhoven, 2011). However, the limited availability of genes investigated through imaging genetics to date might bias our interpretation of the data. In ID, the genes studied thus far are related to mTOR signaling, RAS signaling, and translation repression/regulation, thus functioning in very 'basal' cell signaling pathways in comparison to the genes investigated for ADHD, which regulate the dopamine and serotonin neurotransmitter systems specifically. This could explain the much more widespread cell proliferation/migration defects observed in ID, whereas in ADHD defects seem more specific, e.g. limited to individual neurotransmitter systems and or affecting cell-cell communication more acutely. ASD seems to be intermediate between the other two disorder spectra, but more studies are necessary to substantiate this view. What is already very clear from the available studies, is that the associations of genetic factors are with behavioral traits, and not with the disorders directly (e.g., (Hoogman et al., 2011). Some level of pleiotropy is highly likely, which may also form the basis of comorbidity between the neurodevelopmental disorders.

In general, we found the existing imaging genetics literature for the three neurodevelopmental disorders of our interest lacking in several aspects. Firstly, despite our focus on well-supported candidate genes, several of the selected genes had not been studied at all with MRI in humans. In several additional cases, only single studies were available for different MRI modalities (sMRI, DTI, fMRI), thus limiting the conclusiveness of the reported findings. Secondly, most imaging genetics studies, especially the earlier ones, suffer from being underpowered. The small sample sizes are severely hampering the generalization of findings to the population the samples are meant to represent (Button et al., 2013). Although the endophenotype concept postulates that measures, which mediate

a genetic effect on behavior (including some of those investigated in the imaging genetics studies), should have stronger effect sizes for gene effects than the behavioral/disease measures (Gottesman and Gould, 2003), the sample size of most studies would still have to be considered too small. The problem of limited number of samples becomes evident from e.g. a recent review by Strike and coworkers. They showed that at the most lenient threshold for significance ($\alpha = 0.05$) studies with at least 1,566 participants would be needed to achieve the canonical 80% power threshold to detect a reasonable effect size (0.5% of the phenotypic variance explained) (Strike et al., 2015). Furthermore, recent work raises doubts about whether larger effect sizes can really be expected for neuroimaging (endo) phenotypes, at least for volumetric MRI measures (Franke et al., 2016; Hibar et al., 2015b). Major challenges are the large inconsistency across genetic variants tested and genotype groups compared, differences in study designs and imaging modalities, and the fact that data acquisition and analysis protocols usually were not standardized across studies. Additionally, we observed large inconsistency across studies in the way how genotypic effects were reported and recommend a standardized way of reporting results, e.g. including at least effect estimates and standard errors. Nevertheless, meta-analyses are strongly needed in order to enable definition of robust findings and realistic effect estimates. Therefore, meta-analytic studies would be beneficial for those brain measures covered by multiple studies, as it was shown for the effect of the serotonin transporter 5HTTLPR on amygdala activation (Murphy et al., 2013). Thirdly, to interpret observed links between genes, brain, and behavior properly, one needs to determine, whether a brain (endo)phenotype is really intermediate between a genetic factor and a behavioral outcome, or if it is only an epiphenomenon unrelated to the behavior of interest (Kendler and Neale, 2010; Preacher and Hayes, 2008). Only few studies have really studied this, e.g. by mediation analysis including environmental, behavioral, and/or physiological variables (Klumpers et al., 2014; van der Meer et al., 2015), by applying combinations of different imaging modalities (Kobiella et al., 2011; Zhang et al., 2015b), or by using causal modeling (Sokolova et al., 2015). The results of those studies show that only part of the brain regions showing genotype effects actually do mediate between genetics and behavior, proving the importance of such multilevel investigations. Fourthly, age effects might also be of importance, but have been neglected in most studies. Our own work has shown, for example, that the risk factor for ADHD in *DAT1* differs between children and adults, which resulted in effects of the 9-6 VNTR haplotype on caudate nucleus volume only in adult patients (Onnink et al., 2016). Age effects have also been observed for the 5-HTTLPR variant (Wiggins et al., 2014a). Fifthly, current brain imaging genetics studies often suffer from additional limitations, such as the low ethnic diversity, as most studies included cohorts of only Caucasian origin, and gender imbalance, especially in studies of childhood ADHD and ASD that showed an over-representation of males.

An important additional aspect is that this review enabled us to look at the overlap between studies in healthy individuals and those in patients (case-control designs). An

interaction between genetic variant and diagnosis was indeed observed in some studies (e.g. (Durston et al., 2008; Monuteaux et al., 2008; Wiggins et al., 2012b; Wiggins et al., 2014b). With the available limited amount of evidence it is hard to judge though, whether this is a true difference between patients and healthy individuals, or whether it is simply due to power restrictions in the samples investigated. Recent genome-wide studies investigating the genetics of brain structure as part of the ENIGMA Consortium (Thompson et al., 2014) suggest that effects are largely similar for healthy individuals and those with a psychiatric disorder (Hibar et al., 2015b; Stein et al., 2012). This means, that brain imaging genetics studies with healthy participants can be very informative in discovering related brain correlates and in understanding the biological mechanisms leading to diseases of interest.

Did we overlook important literature through the choices made in our review? We did restrict our selection of genes to study. For ASD, we did not include genes harboring rare genetic variants, while those might result in stronger effect sizes, as observed for the ID genes. However, most of the rare variants linked to ASD have only recently been identified, making the availability of imaging genetics studies (with 10 or more cases) unlikely. A similar argument holds true for our selection of ID genes, where the imaging genetics literature is largely focused on the relatively common disorder subtypes we included in our study. We also restricted our search to MRI-based studies, following a first screen of the literature showing that this was the predominant method used for imaging genetics studies of the neurodevelopmental disorders. Nevertheless, for several genes/variants, also other imaging modalities have been employed, which may provide additional insights. EEG and MEG offer a much higher time resolution than MRI, and may allow investigation of genetic influences on neuronal functioning and oscillation patterns. PET can provide information on (acute) protein availability. Especially the integration of modalities in the study of individual participants can provide deeper insights into mechanisms (e.g. (Kobiella et al., 2011)). Moreover, future studies might want to investigate additional comorbid neurodevelopmental disorders, such as conduct disorder (CD) or obsessive-compulsive disorder (OCD), once robust association of genetic variants with these disorders has been established and investigated in imaging genetics studies.

To summarize, despite the considerable numbers of imaging genetics studies in neurodevelopmental disorders available for review, this field of research should still be considered in its early stages. More genes need to be studied, and individual genes need to be investigated in larger samples, with more hypothesis-generating brain- and phenome-wide methods. Gene-environment interactions and age effects should be taken into account. While we see consistent findings for single genes and variants, gene-wide and gene-set analyses, with polygenic scores explaining more phenotypic variance and thus improving study power (Bralten et al., 2011), are likely to take the stage in the future. Several early examples reviewed here already show the promise of this work (e.g. (Nikolova et al., 2011; Passamonti et al., 2008; Stice et al., 2012). As the genes in such sets often show

different gene expression patterns, (structural and functional) connectivity patterns are likely the best brain phenotypes to be studied with such approaches (see above). In the future, we are also likely to see studies approaching imaging genetics in a different way, by asking the question, whether genes contributing to brain structure/function observed in hypothesis-free, genome-wide approaches also contribute to disease-related phenotypes (Franke et al., 2016). First studies of this kind have been published for schizophrenia (Franke et al., 2016) and obsessive compulsive disorder (OCD) (Hibar et al., 2015a), based on results of findings from the ENIGMA GWAS of brain structure (Hibar et al., 2015b; Stein et al., 2012). To successfully map the biological pathways from gene to disease, imaging genetics studies need to be combined with complementary approaches (Klein et al., in press). Recent examples for this are provided by studies by our own group, in which we investigated effects of ADHD-associated genes for their effects in the fruit fly *Drosophila melanogaster* (Klein et al., 2015; van der Voet et al., 2016), as well as the study by Jia and coworkers, in which the authors identified a genetic variant significantly associated with dysfunctional reward, a cognitive and affective deficit frequently observed in ADHD, then verified gene function in locomotion in the fruit fly model (Jia et al., 2016). In conclusion, although still in its early stages, results from studies available thus far already confirm that the imaging genetics approach is suitable to provide more insight into the link between genes, the brain, and behavior in neurodevelopmental disorders.

Acknowledgements

The authors would like to acknowledge grants supporting their work from the Netherlands Organization for Scientific Research (NWO), i.e. the NWO Brain & Cognition Excellence Program (grant 433-09-229) and the Vici Innovation Program (grant 016-130-669 to BF). Additional support is received from the European Community's Seventh Framework Programme (FP7/2007 – 2013) under grant agreements n° 602805 (Aggressotype), n° 602450 (IMAGEMEND), and n° 278948 (TACTICS), and from the European Community's Horizon 2020 Programme (H2020/2014 – 2020) under grant agreements n° 643051 (MiND) and n° 667302 (CoCA). The work was also supported by grants for the ENIGMA Consortium (grant number U54 EB020403) from the BD2K Initiative of a cross-NIH partnership.

Conflict of interest

None of the authors report conflicts of interest. Barbara Franke discloses having received educational speaking fees from Merz and Shire.

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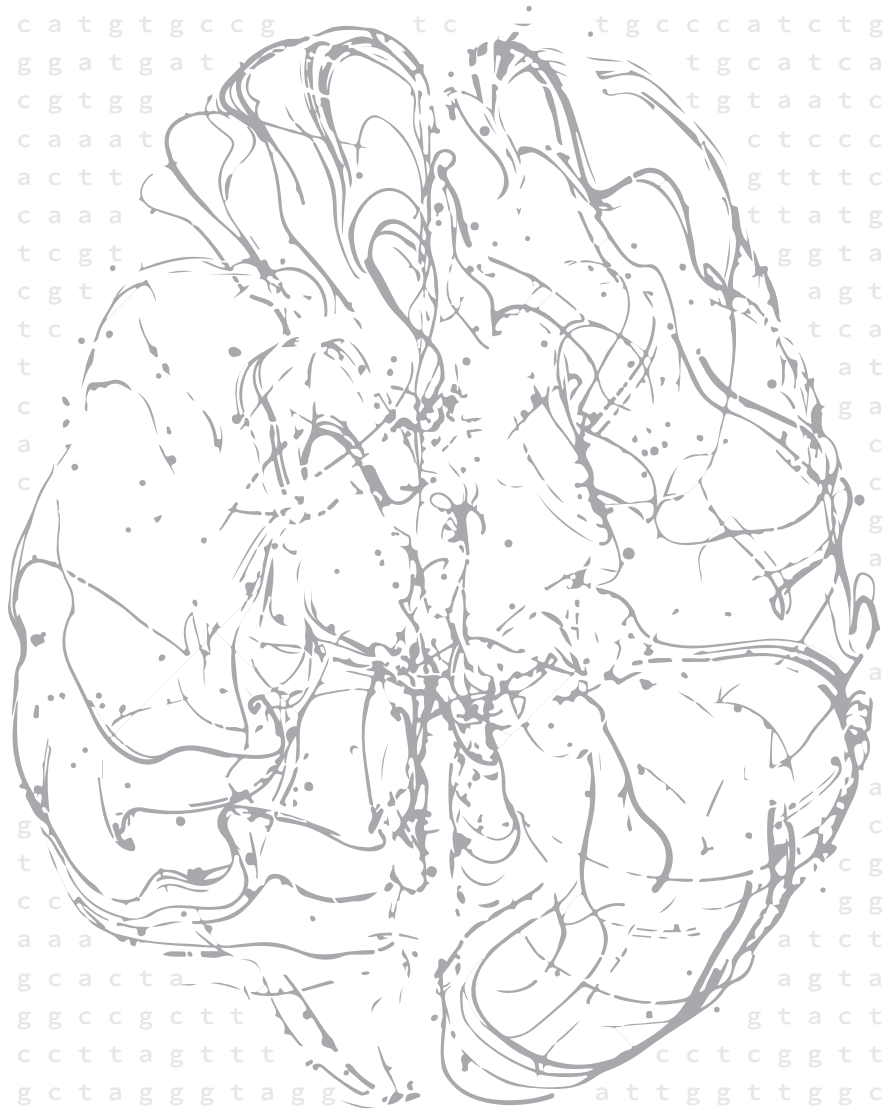

CHAPTER 8

Brain imaging genetics in ADHD and beyond – mapping pathways from gene to disorder at different levels of complexity

Published as:
M. Klein, M. Onnink, M. van Donkelaar, T. Wolfers, B. Harich, Y. Shi, J. Dammers, A. Arias-Vasquez, M. Hoogman, and B. Franke (2017). "Brain imaging genetics in ADHD and beyond - Mapping pathways from gene to disorder at different levels of complexity." Neurosci Biobehav Rev **80**: 115-155.

Abstract

Attention-deficit/hyperactivity disorder (ADHD) is a common and often persistent neurodevelopmental disorder. Beyond gene-finding, neurobiological parameters, such as brain structure, connectivity, and function, have been used to link genetic variation to ADHD symptomatology. We performed a systematic review of brain imaging genetics studies involving 62 ADHD candidate genes in childhood and adult ADHD cohorts. Fifty-one eligible research articles described studies of 13 ADHD candidate genes. Almost exclusively, single genetic variants were studied, mostly focussing on dopamine-related genes. While promising results have been reported, imaging genetics studies are thus far hampered by methodological differences in study design and analysis methodology, as well as limited sample sizes. Beyond reviewing imaging genetics studies, we also discuss the need for complementary approaches at multiple levels of biological complexity and emphasize the importance of combining and integrating findings across levels for a better understanding of biological pathways from gene to disease. These may include multi-modal imaging genetics studies, bioinformatic analyses, and functional analyses of cell and animal models.

Keywords: ADHD, brain imaging genetics, endophenotype, candidate genes, animal models

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a common neurodevelopmental disorder (Faraone et al., 2015). The world-wide prevalence has been estimated at 5% in children and between 2.5 and 4.9% in adults (Polanczyk and Rohde, 2007; Simon et al., 2009). Approximately 55-75% still carry the diagnosis in adulthood or remit only partially displaying several impairments also in adulthood (Faraone et al., 2006). ADHD is characterized by age-inappropriate levels of inattention and/or hyperactivity and impulsivity (Frances, 2000), but the clinical phenotype is heterogeneous (American Psychiatric Association, 2013; Frances, 2000). Severity level and presentation of ADHD can change during a person's lifetime, with adult patients displaying less obvious symptoms of hyperactivity and impulsivity (Buitelaar et al., 2011; Haavik et al., 2010). The phenotypic heterogeneity of the disorder is apparent from a large diversity of psychiatric co-morbidities, frequently seen both in children (Biederman and Faraone, 2005; Gillberg et al., 2004; Lycett et al., 2015; Rappley, 2005; Reinhardt and Reinhardt, 2013) and in adults (McGough et al., 2005; Miller et al., 2007; Ollendick et al., 2008; Sobanski et al., 2007; Wilens et al., 2009).

Identification of ADHD candidate genes

The etiology of ADHD is strongly influenced by genetic factors, as demonstrated by twin and adoption studies (Burt, 2009; Faraone and Mick, 2010; Kotte et al., 2013; Thapar et al., 2013). Heritability estimates range between 70 and 90% (Faraone and Mick, 2010; Larsson et al., 2013b). Despite this substantial heritability, identification of ADHD risk genes has been challenging (Franke et al., 2009; Gizer et al., 2009). One reason for this may be that ADHD has a complex, polygenic genetic background, in which multiple genetic variants (many of them with small effects) contribute to the etiology of the disorder in most patients. Although a substantial fraction of ADHD etiology is due to genes, many environmental risk factors and potential gene-environment interactions are also linked with an increased risk for the disorder (Banerjee et al., 2007; Han et al., 2015). Furthermore, it has been shown that persistent ADHD and its paediatric form are genetically linked, but overlap only partially (Chang et al., 2013).

Multiple molecular genetic studies, employing mostly hypothesis-driven and some hypothesis-free approaches, have been used to identify ADHD risk genes. Because of the high prevalence of ADHD in the population, the search for genetic factors has mainly focused on common genetic variants, which generally have small effect sizes (Li et al., 2014; Neale et al., 2010b).

While many of the individual hypothesis-driven, candidate gene-based association studies have been underpowered, meta-analysis of those studies identified significant associations for common genetic variants in several candidate genes (Faraone et al., 2005; Gizer et al., 2009; Li et al., 2006a). Those are the dopamine and serotonin transporter encoding

genes, *SLC6A3/DAT1* and *SLC6A4/5HTT*, genes coding for the D4 and D5 dopamine receptors, *DRD4* and *DRD5*, a serotonin receptor, *HTR1B*, and the gene for the synaptosomal-associated protein 25, *SNAP25*. Some additional genes (encoding dopamine beta-hydroxylase [*DBH*], adrenoceptor alpha 2A [*ADRA2A*], tryptophan hydroxylase 2 [*TPH2*], and monoamine oxidase A [*MAOA*]) were found suggestively associated with ADHD in meta-analyses (Faraone et al., 2005; Gizer et al., 2009; Li et al., 2006a). In addition, an in depth analysis of 51 genes in a European multisite sample of 674 families with ADHD combined type probands, collected for the International Multisite ADHD Gene project (the IMAGE project), identified associations with ADHD candidate genes, such as *ADRAB2*, *DAT1*, *DRD4*, *TPH2*, and *MAOA* (Brookes et al., 2006a). For more extensive reviews of ADHD candidate genes see references (Banaschewski et al., 2010; Faraone and Mick, 2010; Franke et al., 2012; Hawi et al., 2015; Li et al., 2014).

Genetic linkage studies provided a first possibility to perform hypothesis-free genetic studies in the early 2000s. However, gene identification through linkage analysis has been limited (Banaschewski et al., 2010). A meta-analysis of seven linkage studies revealed a locus on the short arm of chromosome 16 to be relevant for ADHD etiology (Zhou et al., 2008). An interesting candidate gene in the locus is cadherin 13 (*CDH13*), a gene also found in the top-ranks of several genome-wide association studies (Rivero et al., 2015). Linkage analysis also identified the latrophilin 3 (*LPHN3*) gene on chromosome 4, which was subsequently confirmed through association testing (Arcos-Burgos et al., 2010; Ribases et al., 2011). Genome-wide association studies (GWAS) of common single nucleotide polymorphisms (SNPs) have been the main hypothesis-free approach to studying the genetics of ADHD during the last ten years. However, with nine GWASs on ADHD and ADHD-symptoms published to date (Hinney et al., 2011; Lasky-Su et al., 2008b; Lesch et al., 2008; Mick et al., 2010; Neale et al., 2008; Neale et al., 2010a; Sanchez-Mora et al., 2014; Stergiakouli et al., 2012; Yang et al., 2013), no locus has yet been identified that meets genome-wide levels of significance (Li et al., 2014; Neale et al., 2010b), nor has meta-analysis of these studies provided one (Van Hulzen et al., 2016). An interesting genome-wide approach to identify ADHD genes has been the analysis of overlap with other psychiatric disorders. A cross-disorder GWAS across five main neuropsychiatric disorders (schizophrenia [SCZ], bipolar disorder [BD], autism spectrum disorder [ASD], major depressive disorder [MDD], and ADHD) identified five genome-wide significant findings, four of which - in/near the genes *ITIH3*, *AS3MT*, *CACNA1C*, and *CACNB2* - were shared with ADHD (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013).

Following promising results in other psychiatric disorders (Cruceanu et al., 2013; Cukier et al., 2014; Kerner et al., 2013; Purcell et al., 2014), studies of rare variants were also performed for ADHD and were successful in identifying genetic variants related to the disorder (Elia et al., 2010; Lesch et al., 2011; Ramos-Quiroga et al., 2014; Williams et al., 2012; Williams et al., 2010; Yang et al., 2013). Genome-wide analysis of (rare) copy number variants (CNVs) showed an enrichment of rare CNVs in patients with ADHD (Williams et al., 2010), and implicated

the genes *CHRNA7* and *NPY* in ADHD etiology (Lesch et al., 2011; Williams et al., 2012), as well as genes encoding several glutamate receptors (Akutagava-Martins et al., 2014; Elia et al., 2012), and regions on 15q11-15q13 (Valbonesi et al., 2015) and 16p13.11 (Williams et al., 2010). The picture emerging from those initial studies is that the rare variant contribution of ADHD genetics is highly heterogeneous, similar to the common variant contribution. Nevertheless, given the success of CNV studies, exome and whole-genome sequencing are now being used, allowing the identification of rare single nucleotide variants and small insertions/deletions contributing to ADHD etiology. A first study indeed found enrichment of rare variants in a predefined set of 51 candidate genes in adult patients with persistent ADHD (Demontis et al., 2016).

The genetic factors associated with ADHD are distributed across the genome, but tend to be enriched within specific functional categories. By clustering ADHD-related genes within functional networks or pathways, several biological processes have been shown to be involved in the etiology of the disorder. Top-findings of five GWASs in ADHD showed convergence on the biological process of neurite outgrowth (Poelmans et al., 2011). Comparable enrichment analyses revealed that most significantly enriched functions for the ADHD-GWAS association signals were related to nervous system development, neuron projection morphogenesis, oxogenesis, cell-cell communication, glutamatergic synapse/receptor signalling, and multicellular organismal development (Hawi et al., 2015) or neuron projections and synaptic components (Yang et al., 2013), which is consistent with a neurodevelopmental pathophysiology of ADHD. These findings were strengthened by results from a recent study, that used two GWAS datasets to identify pathways associated with ADHD by applying six pathway analysis methods (Mooney et al., 2016). Cross-method convergent results revealed a number of brain-relevant pathways, such as RhoA signaling, glycosaminoglycan biosynthesis, fibroblast growth factor receptor activity, and pathways containing potassium channel genes (Mooney et al., 2016). Another study revealed that CNVs involved in ADHD converge on biologically meaningful gene clusters related to ion channel pathways, organonitrogen compound catabolic processes, and transmembrane transport (Thapar et al., 2015). A combined analysis of ADHD candidate genes, derived both from SNP-based and CNV-based studies, showed that genes involved in biological processes, such as synaptic transmission, catecholamine metabolic processes, G-protein signalling pathways, and cell migration were over-represented among the top-findings of such studies (Cristino et al., 2014). More generally, the genome-wide analysis of five major psychiatric disorders (also including ADHD), supported a role for calcium channel signalling genes for all five disorders, suggesting that genetic variation in calcium-channel activity genes can have pleiotropic effects in the development of psychopathology (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013).

Brain correlates of ADHD

The effects of ADHD genetic risk factors on (aberrant) behaviour are likely to be mediated through effects on cell biology and brain system development and functioning. Several aspects of brain development, structure, function, and connectivity have been found altered in ADHD (Cortese et al., 2012; Glahn et al., 2010; Greven et al., 2015; Hoogman et al., submitted; Mostert et al., 2016; Onnink et al., 2015; Shaw et al., 2007b; Shaw et al., 2012; van Ewijk et al., 2012).

Two indirect neuroimaging techniques that have been used in imaging (genetics) studies in ADHD are positron emission tomography (PET) and single photon emission computed tomography (SPECT). Both are based on the measurement of a radionuclide's decay, during which a positron or a γ-ray is emitted, generating photons. The high sensitivity and limitless penetration depth of PET and SPECT enable imaging to examine metabolic activity, cerebral perfusion, neurotransmitter turnover, and receptor binding potentials within examined brain regions or receptor systems (Rahmim and Zaidi, 2008). Until now, the majority of recent PET studies using ADHD samples have focused on examining differences and changes in neurotransmitter binding and receptor density (Zimmer, 2009). *In vivo* imaging of the dopamine transporter (DAT) is particularly relevant for ADHD, given that DAT is the target of stimulant medications and, subsequently, a target protein for studies of pathophysiology. PET and SPECT studies have been useful in comparing striatal DAT availability between ADHD patients and controls (Jucaite et al., 2005; Ludolph et al., 2008; Spencer et al., 2005). A meta-analysis of nine PET studies revealed that striatal DAT density was 14% higher in patients with ADHD compared to healthy controls (Fusar-Poli et al., 2012). Besides that, striatal density in people with ADHD seems to depend on previous psychostimulant exposure, with lower density in drug-naive subjects and higher density in previously medicated patients (Fusar-Poli et al., 2012). In addition to studies exploring DAT density and binding, Volkow and co-workers examined postsynaptic dopamine receptor availability and found that medication-naïve adults with ADHD showed decreased dopamine D2/D3 receptor availability in the left caudate compared to healthy controls. Following administration of methylphenidate (MPH), the ADHD group demonstrated decreased dopamine activity in the caudate compared with controls (Volkow et al., 2007). One SPECT study investigated D2 receptor availability as a function of MPH therapy in ADHD and concluded that D2 receptor availability is significantly reduced in patients with ADHD in all four regions of the striatum (Ilgin et al., 2001).

Structural magnetic resonance imaging (sMRI) allows to noninvasively characterize the structure of the human brain. With the help of sMRI, the different magnetic properties of brain tissues are used to non-invasively map the spatial distribution of these structural properties of the human brain. Thereby, the different brain tissues (grey and white matter) and cortical and subcortical structures of the brain can be accurately mapped, and different aspects of brain structure can be quantified and compared. In general, sMRI has pointed to total brain volume and total grey matter reductions up to 3-5% in ADHD patients compared

to controls (Castellanos et al., 2002; Greven et al., 2015; Valera et al., 2007). To investigate, whether these reductions are global or regional, several brain regions of interest (ROIs) have been studied. A meta-analysis reported significant volume differences in cerebellar regions, total and right cerebral volume, right caudate, and frontal brain areas (Valera et al., 2007). To investigate the most prominent changes in grey matter intensity, detected by using voxel-based morphometry (VBM) analyses, four meta-analyses have been performed to date (Ellison-Wright et al., 2008; Frodl and Skokauskas, 2012; Nakao et al., 2011). Most consistently, grey matter reductions in the ventrolateral prefrontal/insular-striatal regions, such as the right insula, putamen, globus pallidus, and caudate nucleus have been described in ADHD patients (Ellison-Wright et al., 2008; Frodl and Skokauskas, 2012; Nakao et al., 2011; Norman et al., 2016). A recent study could also show that participants with ADHD had significantly smaller grey matter volume in five clusters located in the precentral gyrus, medial and orbital frontal cortex, and (para)cingulate cortices (Bralten et al., 2015). Unaffected siblings of patients with ADHD showed intermediate volumes, significantly different from controls in four of these five clusters (all except the precentral gyrus), suggesting that the volume reductions are unlikely to be a consequence of disease, but may rather contribute to ADHD etiology (Bralten et al., 2015).

Brain differences observed in ADHD have been hypothesized to be partly attributable to a delay in maturational processes (Castellanos et al., 2002). Indeed, the few longitudinal imaging studies of ADHD patients support this hypothesis: for CT measures, Shaw and co-workers investigated growth trajectories of different points of the cortex and reported that cortical thickness maturation in participants with ADHD lagged behind that of healthy controls of approximately three years throughout the cerebrum, but most prominent in the PFC (Shaw et al., 2007a). In addition, also the SA developmental trajectory was found to be delayed in ADHD, especially in the right PFC (Shaw et al., 2012). Support for the developmental delay hypothesis in ADHD also came from cross-sectional meta-analyses of VBM studies, which found increasing age associated with more normal grey matter values in affected brain areas (Frodl and Skokauskas, 2012). The recent large mega-analysis of subcortical regions across 60 years of the lifespan by the Enhancing NeuroImaging Genetics Through Meta Analysis (ENIGMA) ADHD Working Group extended the delayed maturation theory also to the volumes of most subcortical regions (Hoogman et al., submitted). First of all, they observed significant smaller volumes for the nucleus accumbens, amygdala, caudate, hippocampus, putamen, and intracranial volume (ICV) in ADHD cases relative to controls (Hoogman et al., submitted). Age analyses suggested different brain volume trajectories across age for patients and controls. These results from the cross-sectional lifespan analyses were consistent with the early maturation delay hypotheses of ADHD and hint at delays in brain growth and degeneration across the lifespan (Hoogman et al., submitted; Rubia, 2007).

Next to volumetric differences observed in grey matter, white matter structure has also been found to be altered in ADHD, leading to a potential disorganization of the brain's connectivity. Diffusion tensor imaging (DTI) enables non-invasive investigations of the macrostructural integrity and orientation of white matter fibre bundles. DTI measures the directional diffusion of water molecules along neuronal membranes, allowing to map white matter pathways within the brain. One measure frequently derived from DTI is fractional anisotropy (FA). Anisotropy indicates that diffusion occurs in a directional manner, whereas isotropy indicates diffusion in all directions. Other measures derived from DTI include mean diffusivity (MD), which is an average of axial diffusivity (AD) and the perpendicular diffusivities, and radial diffusivity (RD), which is the average of perpendicular diffusivities, the mode of anisotropy, which is sensitive to crossing fibres, and the apparent diffusion coefficient, which indicates the magnitude of diffusion (Le Bihan, 2003; Le Bihan et al., 2001; Yoncheva et al., 2016). In a meta-analysis comparing DTI findings between patients with ADHD and healthy controls five areas with disturbed microstructural integrity in people with ADHD were highlighted, located in white matter tracts subserving the fronto-striatal-cerebellar neurocircuitry (van Ewijk et al., 2012). Most consistently, studies reported white matter anomalies in the corpus callosum in childhood ADHD (van Ewijk et al., 2014) and adult ADHD (Dramsdahl et al., 2012; Onnink et al., 2015). Although the exact neurobiological meaning is not fully understood, reduced FA in the corpus callosum of adult patients with ADHD was driven by changes in RD rather than AD, suggesting that aberrant myelination is a pathophysiological factor in adult ADHD (Onnink et al., 2015). However, replication from longitudinal studies is still lacking, and the differences between patients and controls seem to be widespread and heterogeneous across studies (van Ewijk et al., 2012).

A method to investigate potential changes in brain activity is functional magnetic resonance imaging (fMRI). FMRI is primarily sensitive to the oxygenation of the blood, the so-called blood-oxygen-level-dependent (BOLD) signal. It measures brain function based on the premise that active cells consume oxygen, thereby causing changes in blood oxygenation and subsequently leading to increased blood flow, although the exact link between cell activation, oxygen saturation, and blood flow is still under debate; for review see (Hillman, 2014). Generally in fMRI, alterations in blood flow after a stimulus (e.g. a certain task) or during a resting state are measured. Comparing anatomical or functional brain measures in individuals with ADHD, their unaffected siblings, and healthy comparison subjects, is one of the best ways to examine the suitability of these neural markers as endophenotypes. With respect to functional brain studies, van Rooij and colleagues (2015c) recently reported a distinction in hemodynamic patters during a stop-signal task between patients with ADHD, their unaffected siblings, and control subjects, suggesting the familial nature of these activation patterns. Thus, inhibition-related neural activation could be considered as a valuable endophenotype for ADHD. Several reviews have provided excellent overviews of cognitive and brain (candidate) endophenotypes for ADHD (del Campo et al., 2012; Gallo

and Posner, 2016; Rommelse et al., 2011). In accordance with those reports, dysregulation of structure and function of the fronto-subcortical-cerebellar pathways that control attention, response to reward, salience thresholds, inhibitory control, and motor behaviour are among the most promising endophenotype candidates, and task-based functional MRI studies in ADHD have largely focused on these neurocognitive domains. More specifically, fMRI studies using inhibitory control, working memory, and attentional tasks in patients with ADHD and healthy comparison subjects have shown underactivation of fronto-striatal, fronto-parietal, and ventral attention networks in the patients (Cortese et al., 2012). The fronto-parietal network mediates goal-directed executive processes, whereas the ventral attention network facilitates reorientation of attention towards salient and behaviourally relevant external stimuli. Meta-analyses of fMRI studies of inhibition and attention revealed that patients with ADHD have consistent functional abnormalities in two distinct domain-dissociated fronto-basal ganglia networks. These include the inferior frontal cortex, supplementary motor areas, and anterior cingulate cortex (ACC) for inhibition and the dorsolateral prefrontal cortex (PFC), parietal, and cerebellar areas for attention processes (Hart et al., 2013). Studies using reward-processing paradigms reported reduced activation of the ventral striatum of participants with ADHD in the anticipation phase of reward relative to controls (Plichta and Scheres, 2014), and differences between patients and controls have also been observed during reward receipt (von Rhein et al., 2015). Additionally, a meta-analysis of fMRI studies of timing reported consistent reductions in activation in typical areas of timing, such as the left inferior frontal gyrus (IFG)/insula, cerebellum, and the left parietal lobe in ADHD patients relative to controls (Hart et al., 2012).

In resting state MRI (rs-fMRI), the temporal correlations in neural activity across anatomically disparate brain regions are analysed to examine functional connectivity based on spontaneous brain activity, neural organization, and circuit architecture. Rs-fMRI studies of ADHD have mainly focused on neural circuits implicated in the disorder, especially the default-mode network (DMN), cognitive control network, and cortico-striato-thalamo-cortical loops (Posner et al., 2014). It was shown that ADHD is associated with less-pronounced or absent anti-correlations between the DMN and the cognitive control network, lower connectivity within the DMN itself, and lower connectivity within the cognitive and motivational loops of fronto-striatal circuits (Posner et al., 2014). A recent study in a large sample of adult participants with ADHD and healthy controls, showed that functional connectivity in the executive control network, and to a lesser extent also the cerebellum network, was stronger in the ADHD group (Mostert et al., 2016). Additionally, hyperactivity/impulsivity symptoms were positively correlated with the connectivity strength in these networks (Mostert et al., 2016).

Functional near-infrared spectroscopy (fNIRS) measures concentration changes of oxygenated, deoxygenated, and total haemoglobin in brain haemodynamics by measuring the absorption of near-infrared light projected through the scalp (Gervain et al., 2011).

Thereby, fNIRS provides an indirect measure of neural activity based on changes in blood oxygenation due to metabolic processes within the cortex. Compared to fMRI, fNIRS is less sensitive to movement artefacts, and since the emitters and detectors can be worn in a cap, functional neural activity can be studied, while the participant is interacting with its environment. This makes fNIRS an ideal tool to study brain development, e.g. in children with ADHD (Vanderwert and Nelson, 2014). FNIRS has greater spatial resolution compared to event-related potential (ERP) or EEG techniques, however, since it is dependent on light penetration and reflection, fNIRS can only examine the cortical surface within 2-3 cm of the scalp (Vanderwert and Nelson, 2014). The majority of fNIRS studies on ADHD investigated children with the disorder. These studies particularly focused on alterations in PFC activity during different experimental paradigms, such as Stroop tasks (Negoro et al., 2010; Xiao et al., 2012), working memory tasks (Schecklmann et al., 2010), the Trail Making Test (Weber et al., 2005), or Go/NoGo paradigms (Inoue et al., 2012; Xiao et al., 2012); they consistently pointed towards an attenuated oxygen metabolism within the frontal lobe (Ehlis et al., 2014). Studies in adult ADHD patients suggest that this hypofunctionality is persistently observed throughout development (Ehlis et al., 2008; Schecklmann et al., 2013).

The functional brain imaging techniques electroencephalography (EEG) and magnetoencephalography (MEG) have also been used for the study of ADHD (genetics). EEG directly measures electrical activity from large populations of cells and therefore offers a very good temporal resolution, far superior to fMRI. However, it has a poor spatial resolution, as electric fields smear as they pass through the skull (Ahmad et al., 2016). Every electric field also has a magnetic field, which can be detected by MEG. The spatial resolution of MEG is slightly better compared to EEG, but MEG only measures information strictly from the sulci, thus it is more limited and misses information (van Diessen et al., 2015). The frequency bands mostly studied in ADHD are theta (θ), alpha (α), and beta (β), either individually, or compared to each other (such as theta/beta power or amplitude ratio). In a resting state, (lower frequency) θ band activity can reflect drowsiness or "cortical slowing". The α band activity is usually observed during eyes closed conditions at rest, particularly in posterior brain regions, and it is negatively associated with central nervous system arousal. In contrast, β band activity generally accompanies mental activity and concentration. The θ/β power ratio has been proposed to capture the relative contributions of two relevant frequency bands for ADHD; however, the true functional significance of this measure remains unknown (Loo and Makeig, 2012). It has been reported that patients with ADHD exhibit increased fronto-central theta (θ) band activity and increased theta-to-beta (θ/β) power ratio during rest compared to non-ADHD controls (Loo and Makeig, 2012). While (limited) discriminant validity of these EEG measures for ADHD has been suggested, significant EEG heterogeneity also exists across ADHD-diagnosed individuals (Clarke et al., 2011). In addition to differences in frequency bands, event-related potential (ERP) studies explored various aspects of brain functioning in ADHD and identified a substantial number of ERP correlates

of ADHD (Johnstone et al., 2013). Robust differences between ADHD patients and healthy controls have been reported in several components related to attention (among others including orienting and vigilance), inhibitory control, and performance monitoring, such as error and reward/punishment processes (Johnstone et al., 2013). MEG studies comparing ADHD patients to healthy controls are scarce and have been geared towards investigating attention-related processes. Alterations in oscillation patterns of brain regions involved in such processes have been observed in patients (Franzen et al., 2013; Heinrichs-Graham et al., 2014; ter Huurne et al., 2013).

Importantly, the brain phenotypes found affected in people with ADHD are often moderately to highly heritable. Findings from twin studies showed that brain structure is under strong genetic control. Additionally, twin studies showed that genetic effects varied regionally within the brain, with high heritability estimates (h^2) for frontal lobe volumes ranging from 0.9 to 0.95, for region-based cortical surface areas ranging from 0.48 to 0.77, and moderate estimates for e.g. the hippocampus (h^2-range = 0.4 – 0.69) (Peper et al., 2007). Surface area was predominantly more heritable than cortical thickness (h^2-range = 0.34 – 0.64) (McKay et al., 2014). Global fractional anisotropy (h^2 = 0.55) as well as radial diffusivity (h^2 = 0.72) of white matter showed high heritability (Kochunov et al., 2015; McKay et al., 2014). Moreover, basal neural activity during a resting state condition has also been shown to be under genetic control, as functional connectivity within the default-mode network as a whole was significantly heritable (h^2 = 0.42) (Glahn et al., 2010). Additional examples for moderate heritabilities of neural activity are e.g. brain activation in the cerebellum and cerebral cortex during working memory tasks (h^2-range = 0.5 – 0.65) (Blokland et al., 2014). Strong genetic determination has also been reported for different psychophysiological brain phenotypes measured by EEG, e.g. (Iacono et al., 2014; Smit et al., 2010), and MEG, where different frequency band heritabilities have been described by van Pelt and coworkers (2012).

Rationale for this review

This review aims to provide a systematic overview of brain imaging genetics studies in ADHD, as brain imaging phenotypes are frequently used as endophenotypes in ADHD research. Endophenotypes (or intermediate phenotypes) have been considered a promising strategy in order to gain more insight into the mechanisms leading from a genetic/biological basis of the disease to the full clinical phenotype (Faraone et al., 2014a). Endophenotypes are (1) those characteristics of a disorder that are linked more closely to its neurobiological substrates than its clinical symptoms (Doyle et al., 2005) and (2) share genetic susceptibility factors with the disorder itself (Gottesman and Gould, 2003). As described above, neuroimaging phenotypes, e.g. derived from sMRI (Hulshoff Pol et al., 2006) and DTI measurements (Jahanshad et al., 2013) are highly heritable. Those brain phenotypes altered in ADHD have therefore been considered key endophenotypes for

the disorder, and investigating the genetic influences on these brain measures has been offered as a way for capturing underlying liability for ADHD (Dresler et al., 2014; Durston, 2010; Wu et al., 2014). Compared to existing reviews of brain imaging genetics studies in ADHD (Dresler et al., 2014; Durston, 2003, 2010; Durston et al., 2009; Wu et al., 2014), this review is more comprehensive by including both childhood and adult ADHD studies, a large spectrum of brain imaging modalities, and by investigating a more complete list of ADHD candidate genes. Beyond the systematic review, we also emphasize the need for additional approaches, describing complementary methods, which provide insight from alternative angles into the biological pathways leading from an ADHD risk gene to disease. Especially, we argue that the integration of methods at different analytical levels (e.g. *in silico*, cell, brain, cognition, and behaviour) is needed to unravel the function of ADHD candidate genes.

Methods

For this review, we selected genes that were previously found associated with ADHD. The selection was based on a recent review of ADHD candidate genes, which described 70 genes that are (with at least some evidence) related to ADHD risk (Li et al., 2014), see **Table 1**. We discarded eight genes, for which we did not find evidence for association with ADHD based on the analysis of genetic variation: *ARVCF, ATP2C2, CPLX4, DNM1, EMP2, IL20RA, MMP7,* and *TRIO* (**Table 1**). On November 28th 2016, we searched for all remaining 62 genes, all brain imaging modalities, and ADHD using PubMed (www.ncbi.nlm.nih.gov/pubmed) with the following search algorithm (example is shown for the *SLC6A3/DAT1* gene): (((*SLC6A3* OR solute carrier family 6 neurotransmitter transporter, member 3 protein human OR DAT1 OR dopamine transporter gene OR dopamine transporter [All fields])) AND (gene* OR genetic* OR imaging genetic OR imaging genetics OR genotype OR polymorphism OR SNP OR single nucleotide polymorphism)) AND (structural magnetic resonance imaging OR functional magnetic resonance imaging OR sMRI OR fMRI OR electroencephalography OR diffusion tensor imaging OR DTI OR resting-state functional magnetic resonance imaging OR rsfMRI OR EEG OR magnetoencephalography OR MEG OR single photon emission computed tomography OR SPECT OR positron emission tomography OR PET OR near-infrared spectroscopy OR NIRS OR volume [Title/Abstract])) AND (ADHD OR Attention-deficit/hyperactivity disorder OR [All fields] NOT "review" [Publication Type]). Titles and abstracts of the retrieved records were evaluated for relevant publications. Studies were required to investigate genetic variants in/near the selected ADHD candidate genes, and only studies including patients with ADHD were included. Review articles, medical hypotheses, non-English articles, and studies on animal models were not considered. The preferred reporting items for systematic reviews and meta-analysis (PRISMA) diagram in **Figure 1** describes the number of articles identified and their classification.

Table 1: Candidate genes containing common variants associated with ADHD, adapted from (Li et al., 2014), which served to select genes for this review.

Gene	Protein	Chr position	References for genes associated with ADHD
ADRA1B	Adrenoceptor alpha1B	5q33.3	(Segurado et al., 2011)[a]; (Hawi et al., 2013a)[a]
*ADRA2A**	Adrenoceptor alpha 2A	10q25.2	(Roman et al., 2003)[a]; (Shiffrin et al., 2013)[b]
ADRA2C	Adrenoceptor alpha 2C	4p16.3	(De Luca et al., 2004)[a]; (Cho et al., 2008)
ADRB1	Adrenoceptor beta 1	10q25.3	(Pascoli et al., 2005)[d]
ADRB2	Adrenoceptor beta 2, surface	5q31-q32	(Lasky-Su et al., 2008a)[a, c]; (Brookes et al., 2006a)
ASTN2	Astrotactin2	9q33	(Lesch et al., 2008) [a]; (Lionel et al., 2011); (Lionel et al., 2014)
BCHE	Butyrylcholinesterase	3q26.1-q26.2	(Lesch et al., 2011)[a]; (Jacob et al., 2013)
BDNF	Brain-derived neurotrophic factor	11p14.1	(Friedel et al., 2005)[a]; (Lee and Song, 2015)[b]
CALY	Calcyon neuron-specific vesicular protein	10q26.3	(Plaisancie et al., 2014)[a]
CCSER1/FAM190A	Coiled-coil serine-rich protein 1	4q22.1	(Lantieri et al., 2010)[a,c]
CDH13	Cadherin 13	16q23.3	(Lesch et al., 2008)[a]; (Neale et al., 2010a)[c]
CHRNA3	Cholinergic Receptor, Nicotinic, Alpha 3	15q25.1	(Polina et al., 2014)[a]
CHRNA4	Cholinergic Receptor, Nicotinic, Alpha 4	20q13.33	(Guan et al., 2009)[a]; (Wallis et al., 2009)
CHRNA7	Cholinergic Receptor, Nicotinic, Alpha 7	5q13.3 spanning *CHRNA7*	(Williams et al., 2012)[a]
CNTF	Ciliary neurotrophic factor	11q12	(Ribases et al., 2008)[a]; (Tzang et al., 2014)
*COMT**	Catechol-O-methyltransferase	22q11.21	(Eisenberg et al., 1999)[a]; (Lee and Song, 2015)
CPLX2	Complexin 2	5q35.2	(Lionel et al., 2011)[a]
DBH	Dopamine beta-hydroxylase (dopamine beta-mono oxygenase)	9q34	(Daly et al., 1999)[a]; (Gizer et al., 2009)[b]
DDC	Dopa decarboxylase (aromatic L-amino acid decarboxylase)	7p12.1	(Ribases et al., 2009)[a]; (Lasky-Su et al., 2008b)[c]
DIRAS2	DIRAS family, GTP-binding RAS-like 2	9q22.32	(Reif et al., 2011)[a]
*DRD1**	Dopamine receptor D1	5q34-q35	(Misener et al., 2004)[a]; (Ribases et al., 2012)

Table 1: Continued.

Gene	Protein	Chr position	References for genes associated with ADHD
DRD2/ ANNK1	Dopamine receptor D2/ Ankyrin repeat and kinase domain containing 1	11q22-q23	(Comings et al., 1991)[a]; (Pan et al., 2015)[b]
DRD3	Dopamine receptor D3	3q13.3	(Guan et al., 2009)[a]; (Wu et al., 2012)[b]
DRD4*	Dopamine receptor D4	11p15	(LaHoste et al., 1996)[a]; (Gizer et al., 2009)[b]; (Wu et al., 2012)[b]
		11p15	(Barr et al., 2001)[a]; (Yang et al., 2008)[d]; (Gizer et al., 2009)[b]
DRD5	Dopamine receptor D5	4p16.1	(Daly et al., 1999)[a]; (Gizer et al., 2009)[b]; (Wu et al., 2012)[b]
FADS2	Fatty acid desaturase 2	11q12.2	(Brookes et al., 2006b)[a]
FTO	Fat mass and obesity associated	16q12.2	(Choudhry et al., 2013)[a]
GDNF	Glial cell derived neurotrophic factor	5p13.1-p12	(Simchon-Tenenbaum et al., 2015)[d]; (Shim et al., 2015)
GPRC5B	G protein-coupled receptor, class C, group 5, member B	16p12	(Albayrak et al., 2013)[a]
GRIN2A	Glutamate receptor, ionotropic, N-methyl D-aspartate 2A	16p13.2	(Turic et al., 2004)[a]
GRM5	Glutamate receptor, metabotropic 5	11q14.3	(Elia et al., 2010)[a]; (Hinney et al., 2011)[c]; (Elia et al., 2012)
GRM7	Glutamate receptor, metabotropic 7	3p26-p25	(Mick et al., 2008)[a,c]; (Elia et al., 2012); (Park et al., 2014)
HES1	Hes family bHLH transcription factor 1	3q28-q29	(Brookes et al., 2006a)[a]; (Lasky-Su et al., 2008b)[c]
HTR1A	5-Hydroxytryptamine (serotonin) receptor 1A, G protein-coupled	5q11.2-q13	(Shim et al., 2010)[a]; (Zuo et al., 2015)[d]
HTR1B	5-Hydroxytryptamine (serotonin) receptor 1B, G protein-coupled	6q13	(Hawi et al., 2002)[a]; (Gizer et al., 2009)[b]
HTR1E	5-Hydroxytryptamine (serotonin) receptor 1E, G protein-coupled	6q14-q15	(Lasky-Su et al., 2008b)[a,c]
HTR2A	5-Hydroxytryptamine (serotonin) receptor 2A, G protein-coupled	13q14-q21	(Quist et al., 2000)[a]; (Lasky-Su et al., 2008a)[c]; (Lasky-Su et al., 2008b)[c]

Table 1: Continued.

Gene	Protein	Chr position	References for genes associated with ADHD
HTR2C	5-Hydroxytryptamine (serotonin) receptor 2C, G protein-coupled	Xq23	(Li et al., 2006b)[a]; (Xu et al., 2009)
HTR3A	5-Hydroxytryptamine (serotonin) receptor 3A, G protein-coupled	11q23.1-q23.2	(Hu et al., 2009)[d]
HTR3B	5-Hydroxytryptamine (serotonin) receptor 3B, G protein-coupled	11q23.1	(Oades et al., 2008)[a]
*LPHN3**	Latrophilin 3	4q13.1	(Arcos-Burgos et al., 2010)[a]; (Hwang et al., 2015)[d]; (Ribases et al., 2011)[d]; (Labbe et al., 2012)[a]
*MAOA**	Monoamine oxidase A	Xp11.4-p11.3	(Payton et al., 2001)[a]; (Liu et al., 2011)[d]
MAOB	Monoamine oxidase B	Xp11.4-p11.3	(Li et al., 2008)[a]; (Ribases et al., 2009)[d]
*NOS1**	Nitric oxide synthase 1	12q24.22	(Reif et al., 2009)[a]; (Franke et al., 2009)[c]; (Weber et al., 2015)[b]
PNMT	Phenylethanolamine N-methyltransferase	17q12	(Brookes et al., 2006a)[a]
PRKG1	Protein kinase, cGMP-dependent, type I	10q11.2	(Neale et al., 2010a)[a]
SLC1A3	Solute Carrier Family 1 (Glial High Affinity Glutamate Transporter), Member 3	5p13	(Turic et al., 2005)[a]; (Elia et al., 2009)
*SLC6A2/NET1**	Solute Carrier Family 6 (Neurotransmitter Transporter), Member 2	16q12.2	(Bobb et al., 2005)[a]; (Hohmann et al., 2015)
SLC6A3/DAT1*	Solute Carrier Family 6 (Neurotransmitter Transporter), Member 3; Dopamine transporter 1	5p15.3	(Cook et al., 1995)[a];(Gizer et al., 2009)[b] (Galili-Weisstub and Segman, 2003)[a]; (Gizer et al., 2009)[b] (Brookes et al., 2006c)[a]; (Gizer et al., 2009)[b]
SLC6A4/5HTT*	Solute Carrier Family 6 (Neurotransmitter Transporter), Member 4; serotonin transporter	17q11.2	(Manor et al., 2001)[a]; (Gizer et al., 2009)[b]; (Landaas et al., 2010)[b]

Table 1: Continued.

Gene	Protein	Chr position	References for genes associated with ADHD
SLC9A9/NHE9	Solute Carrier Family 9, Subfamily A, Member 9	3q24	(de Silva et al., 2003)[a]; (Stergiakouli et al., 2012)[c]; (Mick et al., 2010)[c]
SLC18A2/VMAT2	Solute Carrier Family 18 (Vesicular Monoamine Transporter), Member2	10q25	(Toren et al., 2005)[a]
SNAP25*	Synaptosomal-associated protein, 25kDa	20p12-p11.2	(Brophy et al., 2002)[a]; (Gizer et al., 2009)[b]
SPOCK3	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 3	4q32.3	(Weber et al., 2014)[a]; (Gao et al., 2015)
STX1A	Syntaxin1A	7q11.2	(Sanchez-Mora et al., 2013)[d]
SYP	Synaptophysin	Xp11.23-p11.22	(Brookes et al., 2006a)[a]; (Liu et al., 2013a)[d]
SYT1	Synaptotagmin I	12q21.2	(Guan et al., 2009)[a]; (Lasky-Su et al., 2008a)[c]
TCERG1L	Transcription elongation regulator 1-like	10q26.3	(Neale et al., 2010a)[a,c]
TH	Tyrosine hydroxylase	11p15.5	(Segurado et al., 2011)[a]
TPH1	Tryptophan hydroxylase 1	11p15.3-p14	(Gizer et al., 2009)[b]
*TPH2**	Tryptophan hydroxylase 2	12q15	(Walitza et al., 2005)[a]; (Sheehan et al., 2005)[a]; (Gizer et al., 2009)[b]
VAMP2	Vesicle-associated membrane protein 2 (synaptobrevin 2)	17p13.1	(Gao et al., 2015)[a]

Bold text indicates significant result at $P < 0.05$ in Gizer et al., 2009. [a]Association first reported by. [b]Meta-analysis article. [c]GWAS finding. [d]Association in large sample or validation using animal model. *Gene with at least one case-control imaging genetics study; ADHD = Attention-deficit/hyperactivity disorder, chr = chromosome.

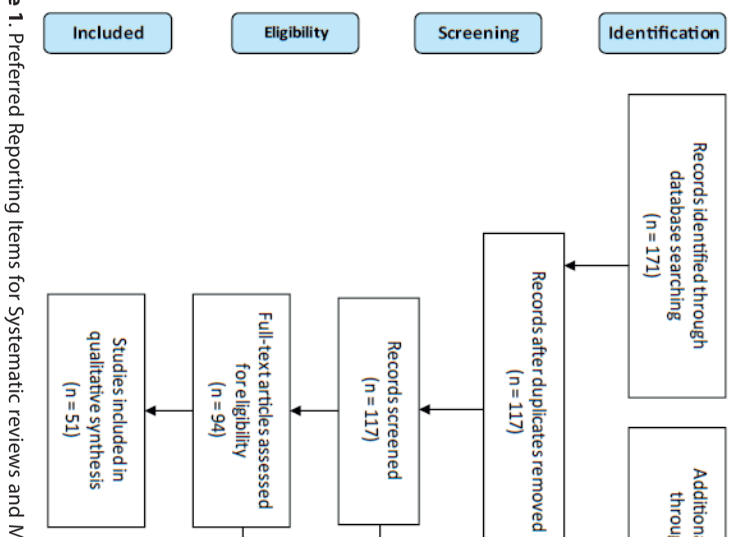

Figure 1. Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA) flowchart of the literature search and study selection for qualitative analysis. Note: see http://www.prismastatement.org for more information in this reporting system.

Results

For 25 out of the 62 ADHD candidate genes, we retrieved a total number of 171 reports linking genetic variation in/near the gene to neuroimaging by using the above mentioned search term in PubMed (**Figure 1**). To those, we added two recent papers from our own group (Sokolova et al., 2015; Van Ewijk et al., in revision) and three additional papers that were missing, from reading the retrieved reports (Albrecht et al., 2014; Dresler et al., 2010; Fernandez-Jaen et al., 2016). After removal of 54 duplicates, we screened 117 records and discarded an additional 66 papers, mostly because they described animal studies, did not include ADHD patients, or did not fulfil our eligibility criteria otherwise. We finally included 51 original research articles on brain imaging genetics studies for 13 ADHD candidate genes (*ADRA2A, COMT, DRD1, DRD4, HTR1B, LPHN3, MAOA, NOS1, SLC6A2/NET1, SLC6A3/DAT1, SLC6A4/5HTT, SNAP25, TPH2*; **Table 2** and **3**). Most of the studies investigated a single gene (32 in Caucasians, 6 in Asians), thirteen studies investigated multiple genes (12 in Caucasians, 1 in Asians). The dopamine transporter gene (*SLC6A3/DAT1*) and the dopamine D4 receptor gene (*DRD4*) were the most frequently studied ADHD candidate genes (**Table 2** and **3**).

Table 2: Overview of imaging genetics studies identified per gene.

Gene	# articles found	# articles discarded	# articles included	References of articles included in review
ADRA2A	4	2	2	(Kim et al., 2010; Park et al., 2013)
BCHE	1	1	0	
BDNF	4	4	0	
CHRNA4	2	2	0	
CHRNA7	1	1	0	
COMT	13	7	6	(Heinzel et al., 2013; Hong et al., 2014; Kabukcu Basay et al., 2016; Shimada et al., 2015; van Rooij et al., 2015b; Villemonteix et al., 2015)
DBH	2	2	0	
DRD1	3	1	2	(Bobb et al., 2005; Shaw et al., 2007a)
DRD2	27	27	0	
DRD3	1	1	0	
DRD4	20	9	12	(Albrecht et al., 2014; Castellanos et al., 1998; Durston et al., 2005; Heinzel et al., 2013; Hong et al., 2014; Loo et al., 2010; Monuteaux et al., 2008; Richards et al., 2016; Schweren et al., 2016; Shaw et al., 2007b; Szobot et al., 2005; Szobot et al., 2011)
DRD5	2	2	0	
GRM5	1	1	0	
HTR1B	1	0	1	(van Rooij et al., 2015a)
LPHN3	2	0	2	(Arcos-Burgos et al., 2010; Fallgatter et al., 2013)
MAOA	3	2	1	(Ko et al., 2015)
MAOB	3	3	0	
NOS1	2	1	2*	(Hoogman et al., 2011; Van Ewijk et al., in revision)
SLC6A2/NET1	7	4	3	(Bobb et al., 2005; Park et al., 2012; Sigurdardottir et al., 2015)
SLC6A3/DAT1	48	22	29*	(Albrecht et al., 2014; Althaus et al., 2010; Bedard et al., 2010; Braet et al., 2011; Brown et al., 2011; Brown et al., 2010; Cheon et al., 2005; Dresler et al., 2010; Drgon et al., 2006; Durston et al., 2005; Durston et al., 2008; Fernandez-Jaen et al., 2016; Fernandez-Jaen et al., 2015; Hong et al., 2014; Hoogman et al., 2013; Krause et al., 2006; Loo et al., 2003; Onnink et al., 2016; Paloyelis et al., 2012; Richards et al., 2016; Rohde et al., 2003; Schweren et al., 2016; Shaw et al., 2007b; Shook et al., 2011; Sokolova et al., 2015; Spencer et al., 2013; Szobot et al., 2005; Szobot et al., 2011; van Rooij et al., 2015b)
SLC6A4/5HTT	10	6	4	(Richards et al., 2016; van der Meer et al., 2016; van der Meer et al., 2015a; van Rooij et al., 2015a)
SNAP25	7	6	1	(Oner et al., 2011)
SYP	1	1	0	
TH	5	5	0	
TPH2	1	0	1	(Baehne et al., 2009)
Total	171	110	66#	

* Papers were added manually (two recent papers from our own group (1x *NOS1*, 1x *SLC6A3/DAT1*) and three additional papers that were missing from the PubMed search (3x *SLC6A3/DAT1*). # This number still includes duplicate articles, since some studies investigated more than one candidate gene. No records were retrieved for the following genes: *ADRA1B, ADRA2C, ADRB1, ADRB2, ASTN2, CALY, CCSER1, CDH13, CHRNA3, CNTF, CPLX2, DDC, DIRAS2, FADS2, FTO, GDNF, GPRC5B, GRIN2A, GRM7, HES1, HTR1A, HTR1E, HTR2A, HTR2C, HTR3A, HTR3B, PNMT, PRKG1, SLC1A3, SLC18A2, SLC9A9, SPOCK3, STX1A, SYT1, TCERG1L, TPH1, VAMP2*.

Table 3: Imaging genetics studies of ADHD candidate genes in ADHD (case-control) samples (for candidate gene list see **Table 1**).

Gene(s)	Variant	Imaging modality	Imaging/cognitive phenotype or task	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
ADRA2A	rs1800544, rs553668	DTI	White matter integrity, FA values	C-allele carriers vs. GG-carriers, T-allele carriers vs. CC-carriers	53 ADHD (9.1)§	rs1800544 C-allele carriers: ↓ FA in right postcentral gyrus. rs553668 T-allele carriers: ↓FA in right middle frontal cortex.	(Park et al., 2013)
	rs1800544	SPECT with ^{99m}Tc-HMPAO	Regional brain perfusion	C-allele carriers vs. GG-carriers	21 ADHD (9.9) ‡ §	C-allele carriers: ↓ perfusion in bilateral orbitofrontal regions.	(Kim et al., 2010)
COMT	rs4680	sMRI (VBM)	Striatum, cerebellum, temporal lobe and IFG volume	Met-carriers vs. Val/Val-carriers	38 ADHD (10.3) 24 HC (10.1)	ADHD Met-carriers: ↓ GM volume in IFG (whole brain level) compared to HC. ADHD Val/Val: ↑ GM volume in right CN (ROI analysis) compared to ADHD Met-carriers and HC.	(Villemonteix et al., 2015)
			GM volume	Met-carriers vs. Val/Val-carriers	17 ADHD (10.3)§ 15 HC (12.8) §	ADHD Met-carriers: ↓ GM volume in left putamen.	(Shimada et al., 2015)
		DTI	FA and RD values	Met-carriers vs. Val/Val-carriers	71 ADHD (10.9) 24 HC (10.8)	ADHD Val/Val: ↓ FA and ↑ RD in the right cingulum (cingulated gyrus) compared to ADHD Met-carriers and HC Val/Val.	(Kabukcu Basay et al., 2016)
DRD4	exon 3 VNTR	sMRI	Superior frontal, middle frontal, anterior cingulate, and cerebellum cortices volumes	ADHD 7R-carriers vs. non-7R-carriers	24 ADHD (38.1) 19 ADHD+BPD (35.8) 20 HC (33.2)	7R- carriers: ↓ volumes of superior frontal cortex and cerebellum cortex compared to non-carriers. No effects in ADHD+BPD or HC.	(Monuteaux et al., 2008)
			TBV, PFC, cerebellum, CN, and pallidum volume	7R-carriers vs. non-7R-carriers	41 ADHD (9.7) 56 HC (17.6)	No volumetric differences between 7R and non 7R-carriers. No group x genotype interactions.	(Castellanos et al., 1998)

Table 3: Continued.

Gene(s)	Variant	Imaging modality	Imaging/cognitive phenotype or task	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
		EEG	β and θ frequency bands during a CPT	7R-carriers vs. non-7R-carriers	340 children (11.1; 304 ADHD) 191 parents (44.3; 80 ADHD)	Childhood 7R-carriers: ↑ frontal θ and ↓ global β2 power. Adult 7R-carriers: similar β2 power in 'eyes closed' condition, but ↓β2 power in 'eyes open' and CPT conditions.	(Loo et al., 2010)
LPHN3	rs2305339, rs734644, rs1397547, rs1397548, haplotype	EEG	Go/No-Go task (CPT)	High risk group (2 copies of risk haplotype = AGCC) vs. low risk group	114 ADHD high risk group (34.85) 102 ADHD low risk group (34.92)	High risk group: ↑ anterior Go-centroid of P300, ↓mean NGA.	(Fallgatter et al., 2013)
	rs6551665, rs1947274, rs2345039 haplotype	Proton magnetic resonance spectroscopy (^{1}H-MRS)	NAA/Cr ratio in striatum, lateral and medial thalamus, cingulate gyrus, and cerebellar vermis	Risk haplotype vs. protective haplotype	Risk haplotype: 13 ADHD, 2 HC Protective haplotype: 1 ADHD, 9 HC Different haplotypes: 8 HC	Risk haplotype carriers: ↓ NAA/Cr in left lateral thalamus, left medial thalamus, right striatum, ↑NAA/Cr in inferior–posterior cerebellar vermis. Carriers of two copies of risk haplotype had lowest levels of NAA/Cr.	(Arcos-Burgos et al., 2010)
MAOA	rs1137070	fMRI	Working memory task	TT-carriers vs. CC-carriers	21 ADHD (23.9) ‡§ 19 HC (25.2) ‡§	ADHD TT-carriers: ↑ activation in left inferior frontal lobe, pars opercularis.	(Ko et al., 2015)
NOS1	Exon 1f-VNTR	DTI	WM integrity, FA and MD values	SS-carriers vs. SL/LL-carriers	178 ADHD (17.6) 122 HC (16.8)	Female SS-carriers: ↑ MD in right parietal WM tracts. Males: no difference between genotype groups. No genotype x diagnostic group interaction.	(Van Ewijk et al., in revision)

Table 3: Continued.

Gene(s)	Variant	Imaging modality	Imaging/cognitive phenotype or task	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
		fMRI	Reward anticipation task/ modified MID task	SS-carriers vs. SL/LL-carriers	63 ADHD (38.3) 41 HC (38.0)	SS-carriers: ↑ activity in VS. No group x genotype interactions.	(Hoogman et al., 2011)
SLC6A2/NET1	rs5569, rs28386840	SPECT with [^{99m}Tc] HMPAO	Cerebral perfusion in response to MPH treatment (8 weeks)	rs5569: GG-carriers vs. GA/ AA-carriers; rs28386840: AA-carriers vs. AT/ TT-carriers	37 ADHD (8.9) §	No differences in baseline clinical assessments or cerebral perfusion based on genotype. rs5569 GG-carriers: After 8 weeks of treatment hyperperfusion in right inferior temporal gyrus and middle temporal gyrus.	(Park et al., 2012)
	rs28386840, rs2242246, rs15534, rs40615	PET with (*S,S*)-[^{18}F] FMeNER-D_2	NET BP_{ND} in thalamus, midbrain with pons, putamen, and cerebellum	Minor allele carriers vs. major allele homozygotes	20 ADHD (30.8) 20 HC (30.4)	rs28386840 and rs2242446 ADHD major allele carriers (A/T): ↑ NET BP_{ND} in the thalamus compared to major allele carrying controls. No difference was detected for the minor allele between groups. rs15534 and rs40615 HC major allele carriers (C/T): ↑ NET BP_{ND} in the cerebellum compared to major allele carrying patients.	(Sigurdardottir et al., 2015)
SLC6A3/DAT1	3′ UTR VNTR	SPECT with[^{99m}Tc] TRODAT-1	Striatal DAT availability	10R/10R-carriers vs. 9R-carriers	29 ADHD (37.7)	No differences in DAT availability between 10R/10R-carriers and 9R-carriers.	(Krause et al., 2006)
		SPECT with [^{123}I]IPT in response to MPH treatment	Basal ganglia DAT density	10R/10R-carriers vs. 9R-carriers	11 ADHD (9.8)§	10R/10R-carriers: ↑ DAT density in basal ganglia.	(Cheon et al., 2005)

Table 3: Continued.

Gene(s)	Variant	Imaging modality	Imaging/cognitive phenotype or task	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
		SPECT with [^{99m}Tc]-ECD in response to MPH treatment	rCBF during a CPT	10R/10R-carriers vs. 9R-carriers	8 ADHD, age range 8-12 ‡	10R/10R-carriers: ↑ rCBF in medial frontal and left basal ganglia areas in response to MPH.	(Rohde et al., 2003)
	3′ UTR, intron 8 VNTR, and haplotype	PET with [^{11}C] altropane	DAT binding in CN	9R-carriers vs. 10R/10R-carriers; 6R-carriers vs. 5R/5R-carriers	34 ADHD (32.8) 34 HC (27.7)	ADHD and HC 9R-carriers: ↑ DAT binding in CN. No association between intron 8 polymorphism or 3′-UTR-intron 8 haplotype with DAT binding.	(Spencer et al., 2013)
	rs2652511, rs2937639 haplotype	PET with [^{11}C] cocaine	Ventral striatal DAT expression	CG-haplotype (rs2652511 C-allele and rs2937639 G-allele carriers) vs. rest	6 ADHD 9 HC	CG-haplotype: No effect on diagnosis. Haplotype was more frequent in individuals with high DAT expression.	(Drgon et al., 2006)
	3′UTR and intron 8 VNTR haplotype	sMRI	Bilateral striatal volumes (nucleus accumbens, CN, and putamen)	Three DAT1 alleles (10/10 genotype, and the haplotypes 10-6 and 9-6)	118 ADHD (35.9) 111 HC (37) 301 ADHD (17.2) 186 HC (16.6) 1718 HC (26.1)	Adult ADHD 9-6 haplotype carriers ↑ 5.9 % larger striatum volume relative to participants not carrying this haplotype (in adult ADHD patients only). Effect was not replicated in adolescent case-control and adult population-based cohort.	(Onnink et al., 2016)
	3′ UTR VNTR	sMRI	Cingulated cortex thickness	10R/10R-carriers vs. 9R-carriers	98 ADHD (10.9)	10R/10R-carriers: ↑ thickness in right cingulated gyrus and right BA 24.	(Fernandez-Jaen et al., 2016)

Table 3: Continued.

Gene(s)	Variant	Imaging modality	Imaging/cognitive phenotype or task	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
	3′ UTR VNTR	sMRI	PFC thickness	10R/10R-carriers vs. 9R-carriers	63 ADHD (10.9)	10R/10R-carriers: ↓ cortical thickness in right BA 46 (lateral PFC). No other prefrontal ROI differed significantly.	(Fernandez-Jaen et al., 2015)
	3′ UTR VNTR	sMRI	CN volume	9R-carriers vs. 10R/10R-carriers	33 ADHD (10.5) 26 HC (10.6)	9R-carriers: ↑ volumes of CN.	(Shook et al., 2011)
	3′UTR and intron 8 VNTR haplotype	fMRI	Striatal activity during reward anticipation task	9-6 haplotype carriers vs. non 9-6 haplotype carriers	87 ADHD (38.3) 77 HC (38)	No differences in striatal activity compared with non 9-6 haplotype carriers nor 9R- and 10R/10R-carriers.	(Hoogman et al., 2013)
					87 ADHD (38.3) 77 HC (38); same as above	Bayesian Constraint-based Causal Discovery (BCCD) algorithm confirmed that there is no direct link between *DAT1* genetic variability and brain activation, but suggested an indirect link mediated through inattention symptoms and diagnostic status of ADHD.	(Sokolova et al., 2015)
			VS and CN activity during reward-predicting cues	*SLC6A3* 10-6 dosage (2 copies vs. <2 copies)	29 ADHD (combined type; 15.8) ‡ 30 HC (15.6) ‡	ADHD: Activation in CN ↓ as number of copies ↑, but in control group reverse was found.	(Paloyelis et al., 2012)
	3′ UTR VNTR	fMRI	Working memory task	9R-carriers vs. 10R/10R-carriers	53 ADHD (35.7) 38 HC (31.2)	9R-carriers: ↓ left medial PFC activation compared to 10R/10R-carriers. Group × genotype interaction showed that 10R/10R-ADHD patients had ↑ activity in pre-SMA/dorsal ACC compared to HC.	(Brown et al., 2011)

Table 3: Continued.

Gene(s)	Variant	Imaging modality	Imaging/cognitive phenotype or task	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
			Go/No-Go task	10R/10R-carriers vs. 9R-carriers	20 ADHD (14.1) 38 HC (13.12)	10R/10R carriers: ↑ activity in frontal, medial, and parietal regions during response inhibition compared to 9R-carriers; ↓error response in the parahippocampal gyrus.	(Braet et al., 2011)
				10R/10R-carriers vs. 9R-carriers	33 ADHD (11.1)	10R/10R carriers: ↑ activity in left striatum, right dorsal premotor cortex, and temporoparietal cortical junction compared to 9R-carriers.	(Bedard et al., 2010)
				9R-carriers vs. 10R/10R-carriers	10 ADHD (14.6)‡ 10 unaffected siblings (14.8)‡ 9 HC (15.3)‡	9R-carriers: ↑ activity in CN and ↓ in cerebellar vermis compared to 10R/10R-carriers. Group × genotype interaction: effect in CN is observed in ADHD and unaffected siblings, but not HC.	(Durston et al., 2008)
			Multi-source interference task	10R/10R-carriers vs. 9R-carriers	42 ADHD (35.4)	9R-carriers: ↓ activity in dorsal ACC compared to 10R/10R-carriers.	(Brown et al., 2010)
		EEG	Go/No-Go task	10R/10R-carriers vs. 9R-carriers	161 ADHD (35.6) 109 HC (35.8)	ADHD 9R-carriers: ↓ NGA (indicating impaired cognitive response control); No genotype effect in control group	(Dresler et al., 2010)
			Feedback-based learning task; measuring ERPs	10R/10R-carriers vs. 9R-carriers	27 ADHD (11.5) 18 PDD (11.3) 20 HC (11.5)	10R/10R-carriers: ↓ Pe to errors and ↓ SPN in anticipation of negative feedback, particularly with learning.	(Althaus et al., 2010)
		EEG, single dose of 10mg MPH (double-blind, placebo-controlled)	Sustained attention and vigilance task	10R/10R-carriers vs. 9R-carriers	27 ADHD (10.1)	10R/10R-carriers: medication-related EEG changes of ↑ central and parietal β power, ↓ right frontal θ power, ↓ θ/β ratios. 9R-carriers: showed opposite pattern.	(Loo et al., 2003)

Table 3: Continued.

Gene(s)	Variant	Imaging modality	Imaging/cognitive phenotype or task	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
SLC6A4/ 5HTT	5-HTTLPR	sMRI (VBM)	GM volume	S-carriers vs. LL-carriers	291 ADHD 78 subthreshold ADHD 332 HC; Average age: 17 years	S-carriers: stress exposure is associated with ↓ GM volume in precentral gyrus, middle and superior frontal gyri, frontal pole, and cingulated gyrus. Association of GxE interaction with ADHD symptom count was mediated by GM volume in frontal pole and anterior cingulated gyrus only.	(van der Meer et al., 2015b)
SNAP25	rs1051312, rs3746544	fNIRS	MPH treatment-related hemodynamic changes during interference condition of Stroop task	rs1051312: TT- vs. TC/CC-carriers, rs3746544: TT- vs. TG/ GG-carriers; 4 groups for interaction analysis	15 ADHD (26.1) 16 ADHD (9.7)	rs1051312 TT-carriers: ↓ right [HHb] with treatment rs3746544 TT-carriers: changes in genotype right [HbO2] and [HHb] as well as left [HHb]. Interaction analysis - participants with rs1051312 CC/ TC and rs3746544 GG/TG: ↑ right [HHb] with MPH use. Interaction analysis - participants with rs1051312 TT and rs3746544 TT or rs1051312 TT and rs3746544 GG/TG: ↓ right prefrontal [HHb] with MPH use.	(Oner et al., 2011)
TPH2	rs4570625, rs11178997	EEG	Go/No-Go task (CPT)	rs4570625: GG- vs. T-carriers, rs11178997: TT- vs. TA-carriers	124 ADHD (34.7) 84 HC (34.8)	rs11178997 TT-carriers and rs4570625 GG-carriers: ↓ NGA in both HC and ADHD. No group x genotype interactions.	(Baehne et al., 2009)
SLC6A3/DAT1, ***DRD4***	3' UTR VNTR, exon 3 VNTR	SPECT with [^{99m}Tc] TRODAT-1 in response to MPH treatment	Striatal DAT binding potential	10/10R-carriers vs. 9R-carriers; 7R-carriers vs. non-7R-carriers; 10/10R+7R-carriers vs. rest	17 ADHD/SUDs, age range 15-21 years ‡	10/10R-carriers: no effect on DAT occupancy after MPH treatment. 7R-carriers: no effect on DAT occupancy after MPH treatment. 10/10R+7R-carriers: ↓ DAT occupancy after MPH treatment in right and left CN and putamen.	(Szobot et al., 2011)

Table 3: Continued.

Gene(s)	Variant	Imaging modality	Imaging/cognitive phenotype or task	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
		SPECT with [^{99m} Tc] ECD	rCBF during a CPT	10/10R-carriers vs. 9R-carriers; 7R-carriers vs. non-7R-carriers; 10/10R+7R-carriers vs. rest	34 ADHD (11.6) ‡	10/10R-carriers: no effect on rCBF. 7R-carriers: no effect on rCBF. 10/10R+7R-carriers: ↑ rCBF in right middle temporal gyrus area.	(Szobot et al., 2005)
		sMRI	PFC GM and CN volume	9R-carriers vs. 10R/10R-carriers, 4R/4R-carriers vs. rest	26 ADHD (12.1) 26 unaffected siblings (11.6) 20 HC (10.7); all ‡	*SLC6A3* ADHD 10/10R-carriers: ↓ CN volumes *DRD4* unaffected siblings 7R-carriers: ↑ prefrontal GM volume. No effects on CN, or TBV. No interactions between ADHD status and genotype.	(Durston et al., 2005)
	3'UTR and intron 8 VNTR haplotype, exon 3 VNTR	sMRI	Striatum, frontal cortex, and hippocampus volumes	10-6 haplotype carriers vs. non-10-6 haplotype carriers, 7R-carriers vs. non-7R-carriers	316 ADHD (17.2) 187 HC (16.5)	*SLC6A3* 10-6 haplotype-carriers: ↓ left striatal volume, irrespective of treatment. *DRD4* 7R-carriers: frontal cortex volume is associated with stimulant treatment at younger age.	(Schweren et al., 2016)
SLC6A3/DAT1, ***DRD4, SLC6A4/5HTT***		sMRI	Total GM, caudate, and putamen volume	9R-carriers vs. rest, 7R-carriers vs. rest, S-allele-carriers vs. rest	368 high ADHD severity (17.3) 374 low ADHD severity (16.8)	For total GM, differential age effects were found for *SLC6A3* 9R- and *SLC6A4* L/L carriers, depending on the amount of positive peer affiliation. For putamen volume, *DRD4* 7R- carriers and *SLC6A3* 10/10 homozygotes showed opposite age relations. Results were independent of ADHD severity.	(Richards et al., 2016)

Table 3: Continued.

Gene(s)	Variant	Imaging modality	Imaging/cognitive phenotype or task	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
SLC6A3/DAT1*, *DRD4*, *DRD1	3' UTR VNTR, exon 3 VNTR, rs4532	sMRI; longitudinal study (mean follow-up, 6 years)	Cortical thickness	9R-carriers vs. 10R/10R-carriers, 7R-carriers vs. non-7R-carriers, C-allele carriers vs. non-C-allele carriers	105 ADHD (10.1; 13.1; 15.9) 103 HC (10.0; 12.4; 14.4)	*SLC6A3* 9R-carriers: No effect on cortical development. *DRD4* 7R-carriers: thinner right orbitofrontal/ inferior prefrontal and posterior parietal cortex. ADHD 7R-carriers: distinct trajectory of cortical development; normalization of right parietal cortical region. *DRD1*: No effect of genotype on clinical outcome or cortical development.	(Shaw et al., 2007b)
SLC6A3/DAT1*, *DRD4*, *COMT	3' UTR VNTR, exon 3 VNTR, rs4680	DTI	WM integrity, FA values	9R-carriers vs. 10R/10R-carriers; 4R/4R-carriers vs. rest; Met-carriers vs. Val/Val	58 stimulant- and atomoxe-tine-naïve ADHD (8.7) §	*SLC6A3* 9R-carriers: no effect on WM integrity *DRD4* 4R/4R-carriers: no effect on WM integrity. Met-carriers: ↓ Network of WM connections linking 18 brain regions	(Hong et al., 2014)
SLC6A3/DAT1*, *COMT	3'UTR and intron 8 VNTR haplotype, rs37020, rs460000, rs4680	fMRI	Stop-signal task	10-6 haplotype-carriers vs. non-10-6 haplotype-carriers; rs37020: CC vs. rest; rs460000: GG vs. rest; Val/Val vs Met-carriers	185 ADHD (17.3) 111 unaffected siblings (17.3) 124 HC (16.5)	No genotype x ADHD interaction effects. *SLC6A3* 10-6 haplotype-homozygotes: ↑ activity related to successful stop-trials in pre-supplementary motor areas, ↓ activity in superior frontal and temporal pole areas. rs37020 AA-carriers: ↓ activity during failed stop-trials in lFG, pre-supplementary motor areas, and post-central gyrus. rs4680 Val/Val-carriers: ↓ activity during successful stop-trials in thalamus, frontal pole, and left IFG; ↑ activity in hippocampus during failed stop-trials.	(van Rooij et al., 2015b)

Table 3: Continued.

Gene(s)	Variant	Imaging modality	Imaging/cognitive phenotype or task	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
SLC6A3/DAT1*, *DRD4	3'UTR and intron 8 VNTR haplotype, exon 3 VNTR	EEG	CPT	10-6 haplotype-carriers vs. non-10-6 haplotype-carriers; 7R-carriers vs. non-7R-carriers	94 ADHD; 31 HC ; age range 8-16 ; all ‡	*SLC6A3* 10-6 haplotype-carriers: ↑ activity related to inhibitory response control (Nogo-P3) *DRD4* 7R-carriers: ↓ activity related to attentional orienting (Cue-P3) and cognitive or response preparation (CNV). No genotype x ADHD interactions.	(Albrecht et al., 2014)
DRD4*, *COMT	exon 3 VNTR, rs4680	EEG	Go/No-Go task	7R-carriers vs. non-7R-carriers; Val/Val vs. Val/ Met vs. Met/ Met-carriers	181 ADHD (35.3, age range: 18-60) 114 HC	Single genes and diagnosis had no effect on neural correlates of prefrontal response control (NGA). *DRD4* VNTR and *COMT* SNP epistatically interacted on NGA.	(Heinzel et al., 2013)
DRD1*, *SLC6A2/NET1	rs4532 and rs265981, rs998424 and rs3785157	sMRI	TCV, volumes of total GM and WM, CN, cerebellum, frontal, temporal, parietal lobes	2 and 3 genotype groups per SNP	114 ADHD (9) 79 HC (16)	*DRD1* and *NET1* SNPs: No genotype effects on GM or WM volume and no group x genotype interactions.	(Bobb et al., 2005)
SLC6A4/5HTT, HTR1B	5-HTTLPR, rs6296	fMRI	Stop-signal task	3 genotype groups per variant	184 ADHD (17.3) 111 unaffected siblings (17.3) 124 HC (16.5)	*SLC6A4* SS-genotype group: ↓ activation in frontal nodes and ↑ activation in posterior nodes. *HTR1B genotype:* associated with differential activation in anterior cingulate, occipital, inferior temporal, and cerebellar regions during successful stop trials. No associations between *SLC6A4* and *HTR1B* variants and ADHD or ADHD-related neural activation.	(van Rooij et al., 2015a)

Table 3: Continued.

Gene(s)	Variant	Imaging modality	Imaging/cognitive phenotype or task	Genotype groups compared	Samples size (mean age in years)	Primary results (main effect of genotype)	Reference
SLC6A4/5HTT, ***NR3C1***	5-HTTLPR, rs6189, rs6198	sMRI	GM volume	S-allele carriers vs. rest; NR3C1 risk haplotype-carriers (rs6189G and rs6198G) vs. rest	539 ADHD, unaffected siblings, and HC combined (17.2)	NR3C1 risk haplotype-carriers: ↑ positive relation between stress exposure and ADHD severity; which was stronger for *SLC6A4* L-allele homozygotes. Interactions were reflected in GM volume of cerebellum, parahippocampal gyrus, intracalcarine cortex, and angular gyrus.	(van der Meer et al., 2016)

BP = binding potential, BP_{ND} = nondisplaceable binding potential BPD = bipolar disorder, CN = caudate nucleus, CPT = continuous performance test, dACC = dorsal anterior cingulated cortex, DAT = dopamine transporter, DTI = Diffusion tensor imaging, ECD = ethyl-cisteinate-dimer, EEG = electroencephalography, ERP = event related potential, FA = fractional anisotropy, fMRI = functional magnetic resonance imaging, fNIRS = functional near-infrared spectroscopy, HC = healthy control, IFG = inferior frontal gyrus, NAA/Cr = ratio of N-acetylaspartate to creatine, NAcc = nucleus accumbens, NGA = NoGo-anteriorization, PDD = Pervasive Developmental Disorder, Pe = error-related positivity, PET = positron emission tomography, PFC = prefrontal cortex, pre-SMA = pre-supplementary motor area, rCBF = regional cerebral blood flow, RD = radial diffusivity, ROI = region of interest, sMRI, structural magnetic resonance imaging, SPECT = single-photon emission tomography, SPN = stimulus-preceding negativity, SUD = substance use disorder, TBV = total brain volume, TCV = total cerebral volume, UTR = untranslated region, VBM = voxel-based morphometry, VNTR = variable number tandem repeat, VS = ventral striatum, 4R = 4 repeat allele, 5R = 5 repeat allele, 7R = 7 repeat allele, 9R = 9 repeat allele, 10R = 10 repeat allele, [HHb] = deoxyhemoglobin, [HbO2] = oxygenated hemoglobin, [^{99m}Tc] = technetium-99; ^{1}H-MRS = Proton magnetic resonance spectroscopy, [^{99m}]Tc-HMPAO = technetium-^{99m} hexamethylpropylene amine oxime, † only females, ‡ only males; § Asian sample

Findings for the dopamine transporter gene (*SLC6A3, DAT1*)

The gene *DAT1* (official name *SLC6A3*) codes for a solute carrier protein (DAT) responsible for the reuptake of dopamine from the synaptic cleft into the presynaptic neuron, representing a primary mechanism of dopamine regulation in the striatum (Ciliax et al., 1999). The most widely studied polymorphism in *SLC6A3/DAT1* is a variable number of tandem repeat (VNTR) sequence in the 3' untranslated region (3'UTR) that is 40 base pairs (bp) in length. Most common alleles are those with 9 and 10 repeats (9R and 10R). Additionally, a 30 bp VNTR in intron 8 of the gene (most common alleles with 5 and 6 repeats [5R and 6R]), is sometimes studied together with the 3'UTR VNTR as a haplotype. The 10R/10R genotype of the 3'UTR VNTR and the 10-6 haplotype of the two VNTRs are thought to be risk factors for childhood ADHD (Asherson et al., 2007; Brookes et al., 2006a). In contrast, the 9R/9R genotype and the 9-6 haplotype are associated with persistent ADHD (Franke et al., 2010). This suggests a differential association of the gene with ADHD depending on age, and a role of *DAT1* in modulating the ADHD phenotype across the lifespan. In addition to the VNTRs, several SNPs in *DAT1* have been studied for their effect on ADHD and/or brain phenotypes.

Two studies performed PET to study the role of *DAT1* genotype on DAT availability, one using ^{11}Altropane as the ligand and one ^{11}Cocaine. In an early study investigating a very small sample of 6 patients with ADHD and 9 controls, Drgon and colleagues studied a haplotype of two SNPs (rs2652511, rs2937639) in the 5' regulatory region of the *SLC6A3/DAT1* gene, and found the CG-allele associated with ventral striatal *in vivo* DAT availability independent of diagnosis; this finding was confirmed through investigation of striatal DAT expression in post-mortem brain samples (2006). Spencer and coworkers observed that, in adults, the 9R genotype of the 3'UTR VNTR increased DAT binding in caudate nucleus both in patients and healthy controls, whereas the intron 8 VNTR and a haplotype of both variants were not associated with DAT binding (2013); (**Table 3**).

Four SPECT studies, using different ligands, investigated the effect of the 3'UTR VNTR on DAT availability (**Table 3**). Two early case-only studies, both in children with ADHD (n=8 and 11, respectively), showed that basal ganglia DAT density was increased (Cheon et al., 2005) and that the regional cerebral blood flow (rCBF) was larger in medial frontal and left basal ganglia during a continuous performance task (CPT) in response to MPH treatment in 10R/10R-homozygotes compared to 9R-carriers (Rohde et al., 2003). A somewhat larger study in adults with ADHD did not identify a difference in striatal DAT availability between 10R/10R-homozygotes and 9R-carriers (Krause et al., 2006). Another SPECT study in boys with ADHD observed a genotypic effect of the 3'UTR VNTR variant increasing rCBF during a CPT only in the presence of risk alleles at both *SLC6A3/DAT1* (10R/10R) and *DRD4* (7R); this effect was present in the right middle temporal gyrus, an area associated with working memory and selective attention (Szobot et al., 2005); (**Table 3**). An interaction effect between the two polymorphisms was subsequently also shown by the same group in adolescent patients with ADHD plus substance use disorder (Szobot et al., 2011); (**Table 3**). In this case, participants

homozygous for the *SLC6A3/DAT1* 10R-allele and carrying the *DRD4* 7R-allele exhibited decreased DAT occupancy after MPH treatment in the right and left caudate nucleus and putamen (Szobot et al., 2011). A recent meta-analysis, including healthy subjects and patients with different psychiatric disorders including ADHD, assessed the association of the 3'UTR variant with DAT availability (Faraone et al., 2014b). The PET studies provided significant evidence that the 9R-allele was associated with increased DAT availability in human adults, independent of the diagnostic status. The SPECT studies were highly heterogeneous, but when the analysis was limited to the most commonly used ligand, stratification by affection status dramatically reduced heterogeneity and revealed a significant association of the 9R allele with increased DAT availability for healthy subjects. The authors concluded that in humans, the 3'UTR polymorphism of *SLC6A3/DAT1* regulates dopamine activity in the striatal brain regions independent of the presence of neuropsychiatric illness.

Eight sMRI studies for *SLC6A3/DAT1* were performed thus far (**Table 3**). Two studies reported a smaller volume of the striatal caudate nucleus in homozygotes for the 10R allele when compared to children with the 9R/10R genotype (Durston et al., 2005; Shook et al., 2011). While Durston and coworkers found this effect to be most pronounced in children with ADHD, rather than their unaffected siblings or healthy comparison subjects, the overall genotype effect was independent of diagnosis. Two recent studies investigated cortical thickness in children and adolescents with ADHD. Fernández-Jaén and colleagues suggested that thickness of the lateral PFC and the cingulated cortex might be influenced by the presence of the 10R-allele (2016; 2015). Interestingly, homozygotes for the 10R allele showed increased thickness in the right cingulated gyrus (Fernandez-Jaen et al., 2016), but decreased cortical thickness in the lateral PFC (Fernandez-Jaen et al., 2015). In addition, a large observational study in an adolescent cohort revealed that irrespective of stimulant treatment, left striatal volume was reduced in participants with ADHD carrying one 10-6 haplotype (Schweren et al., 2016). A recent cross-sectional sMRI study included three cohorts (a childhood/adolescent ADHD case-control sample, an adult ADHD case-control sample, and an adult population-based sample) and showed that only in the adult ADHD case-control cohort, carriers of the *DAT1* adult ADHD risk haplotype 9-6 had a 5.9% larger striatum volume relative to participants not carrying this haplotype. The effect varied by diagnostic status, with the risk haplotype affecting striatal volumes only in patients with ADHD (Onnink et al., 2016). A longitudinal study did not reveal any effect of *SLC6A3/DAT1* genotype on cortical thickness (Shaw et al., 2007b), consistent with the predominant expression of *DAT1* in subcortical (striatal) structures (and cerebellum). A recent gene-environment (GxE) interaction study reported differential age effects for *SLC6A3/DAT1* 9R-allele carriers for total grey matter volume and for *SLC6A3/DAT1* 10R-allele homozygotes on putamen volume, depending on positive peer affiliation (Richards et al., 2016) (**Table 3**). These findings were independent of ADHD severity. The presence of such differential age-dependent GxE effects might explain the diverse and sometimes opposing results of genetic and environmental

effects on brain phenotypes (Richards et al., 2016). A single DTI study was performed in a sample of children with ADHD to assess *DAT1*'s effect on structural connectivity (**Table 3**). However, the investigated 3'UTR VNTR genotype did not appear to affect white matter integrity (Hong et al., 2014).

In total, nine fMRI studies were performed to investigate the effect of *SLC6A3/DAT1* genotype on brain activity related to different tasks, most often examining reward processing and different aspects of executive functioning (**Table 3**). The studies included childhood, adolescent, and adult ADHD samples. Three fMRI studies investigated the role of the *SLC6A3/DAT1* VNTR haplotype using reward-processing paradigms. Reward processing is altered in ADHD, and meta-analysis has shown that activation of the ventral striatum in anticipation of reward is lower in patients with ADHD than in controls (Plichta and Scheres, 2014). In a study in male adolescents, the activation of the caudate nucleus within the ventral striatum was found reduced in the ADHD group as the number of 10-6 haplotype copies increased (Paloyelis et al., 2012). A sizeable study in adult ADHD cases and controls, on the other hand, found no effect of *SLC6A3/DAT1* haplotype on striatal activity (Hoogman et al., 2013). The latter dataset was re-analyzed using a Bayesian constraint-based causal discovery algorithm; this analysis suggested that any links between the genetic haplotype in *DAT1* and reward anticipatory brain activity may be indirect only, mediated through inattention symptoms (Sokolova et al., 2015).

In studies of response inhibition, tested through a Go/No-Go task in children and adolescents, the 10R/10R genotype was found to be linked to higher (Bedard et al., 2010), but also lower (Durston et al., 2008) striatal activation. Interestingly, Durston and colleagues observed genotypic effects in the caudate nucleus in the patients and their unaffected siblings, but not in healthy controls (2008). Beyond the striatum, *SLC6A3/DAT1* genotype effects were also found in additional brain regions, such as during cerebellar activation in children with ADHD (Durston et al., 2008), and in frontal, medial, and parietal regions, where activity was increased during response inhibition in adolescents homozygous for the 10R allele (Braet et al., 2011). Increased activity in the (temporo-)parietal regions in homozygous carriers of the 10R-allele was also observed in a second study, in addition to increased activity in the right dorsal premotor cortex (Bedard et al., 2010). The effects of the *SLC6A3/DAT1* haplotype and rs37020 genotype on neural activation during response inhibition have been investigated as well (van Rooij et al., 2015b) (**Table 3**). Homozygous carriers of the *SLC6A3* 10-6 haplotype exhibited increased activity related to successful stop-trials in pre-supplementary motor areas and reduced activity in superior frontal and temporal pole areas, whereas homozygous carriers of the rs37020 A-allele showed reduced activity during failed stop-trials in the IFG, pre-supplementary motor areas, and post-central gyrus (van Rooij et al., 2015b). Despite these wide-spread effects on neural activation changes of the response inhibition network, these changes were independent of ADHD diagnostic status (van Rooij et al., 2015b). As expression of *DAT1* is limited outside of striatum and cerebellum,

these latter effects are likely due to direct or indirect connections between the regions of gene expression and the rest of the brain.

A working memory task in adult participants elicited increased activity in the dorsal ACC in patients homozygous for the 10R allele, whereas this genotype caused reduced activity in controls (Brown et al., 2011). Additionally, the authors showed a marginal association of the *SLC6A3/DAT1* genotype with task-related suppression in the left medial PFC (Brown et al., 2011). Also in a multi-source interference task, the 10R/10R homozygotes had increased activity in the dorsal ACC compared to carriers of the 9R-allele (Brown et al., 2010). The dorsal ACC is thought to play a crucial role in numerous cognitive control functions including attention modulation, competition monitoring, complex motor control, novelty, error detection, working memory, anticipation of cognitively demanding tasks, and the modulation of reward-based decision making (Shenhav et al., 2013). Functional abnormalities associated with the dorsal ACC have been repeatedly reported in ADHD (Cao et al., 2009; Castellanos et al., 2008; Tamm et al., 2004; Tian et al., 2006; Zang et al., 2007), and the results above suggest that these effects might be most pronounced in 10R-allele homozygotes, which constitute approximately 71.9% of the Caucasian population (Doucette-Stamm et al., 1995).

No studies in patients have yet investigated effects of *DAT1* genotype on functional brain connectivity assessed through resting state fMRI. A single study in healthy participants using seed-based analysis revealed that carriers of the 9R-allele showed stronger connectivity between dorsal caudate nucleus and insula, dorsal ACC, and dorsolateral prefrontal regions, as well as between ventral striatum and centrolateral prefrontal cortex, suggesting widespread effects of the *SLC6A3/DAT1* genotype on functional connectivity of striatal structures with the rest of the brain (Gordon et al., 2015).

Four EEG studies in ADHD childhood samples investigated the effects of *SLC6A3/DAT1* variation (**Table 3**). One study examined the influence of the common 9R and 10R alleles on prefrontal brain functioning and cognitive response control in participants with adult ADHD and healthy controls (Dresler et al., 2010). By means of a Go-NoGo task (CPT) they inspected a neurophysiological marker of cognitive response control (NoGo-anteriorization, NGA). The NGA is a endophenotypic marker of prefrontal functioning, reflecting neural correlates of both response inhibition and execution in a Go-NoGo test situation (Fallgatter and Strik, 1999). It is a topographic event-related potential parameter quantifying the brain's electrical field frontalization during motor inhibition (NoGo, when compared with response execution Go). As such, the NGA reflects "NoGo" activation of the medial PFC (anterior cingulate cortex, ACC) (Fallgatter et al., 2002). The NGA and the electrical field frontalization during NoGo trials have been found reduced in patients with ADHD (as well as those with schizophrenia) compared with healthy controls, reflecting diminished activation of the medial PFC in these patient groups (Fallgatter et al., 2005; Fallgatter and Muller, 2001). In the study of *SLC6A3/DAT1* genotype carriers of the 9R-allele within the ADHD group showed significantly reduced

NGA, whereas no influence of genotype was observed in the control group (Dresler et al., 2010). A second EEG study investigated the effect of the 10-6 haplotype on response control, by using a CPT task in a childhood case-control sample (Albrecht et al., 2014). Independent of ADHD diagnosis, 10-6 haplotype-carriers exhibited elevated brain activity related to inhibitory response control (Albrecht et al., 2014). Another study investigated the effect of the *SLC6A3/DAT1* 3'UTR genotype on neurophysiological correlates of performance monitoring by measuring event-related potentials (ERPs) during a feedback-based learning task. The authors showed that 10R/10R homozygotes had a smaller error-related positivity (Pe) response to errors and a smaller stimulus-preceding negativity (SPN) in the anticipation of negative feedback, especially with learning (Althaus et al., 2010), suggesting that *SLC6A3/DAT1* genotype influences a system that is sensitive to aversive stimuli and their conscious processing (Althaus et al., 2010). The third EEG study investigated MPH medication-related changes on cortical power spectra during a sustained attention and vigilance task in a patient-only sample. ADHD patients have been shown to have increased frontocentral θ band activity and increased θ/β ratio compared to non-ADHD controls during rest (Loo and Makeig, 2012). Generally, the β band activity has been associated with attentional arousal, and it has been suggested that the θ/β ratio may reflect increased impulsivity and difficulty negotiating the speed-accuracy trade-off (faster speed but poorer performance) in ADHD (Loo and Makeig, 2012). In their study of DAT1 genetic variation during MPH treatment, the authors reported that those children with ADHD homozygous for the 10R-allele, exhibited medication-related cortical changes of increased central and parietal β power, decreased right frontal θ power, and lower θ/β ratios; 9R carriers showed the opposite pattern (Loo et al., 2003).

Summarizing, sMRI studies (as well as PET/SPECT by definition) find *SLC6A3/DAT1* genotype effects mainly in regions, in which the gene is preferentially is expressed. These local effects can have widespread consequences on brain activity, as fMRI and EEG studies reveal. However, it is still unclear how the PET/SPECT findings, such as high DAT binding and density in nucleus accumbens and dorsal striatum (Kuczenski and Segal, 2002; Segal and Kuczenski, 1997) or the differences observed in brain activity are related to structural volume differences in the specific brain regions of ADHD patients. Therefore, studies could benefit from combining different imaging methodologies. The effects of genotype are often observed independent of diagnostic status, as would be expected for bona fide risk factors. Additionally, credibility of most of the existing studies can be questioned, since many of them are limited by their small sample sizes (Mier et al., 2010; Munafo et al., 2008). The actual neuroanatomical and brain activity-based mechanisms by which *SLC6A3/DAT1* genotype increases ADHD risk remain to be clarified, as the results of studies published thus far are too patchy (and based on too small samples) to allow a coherent story to be defined. Moreover, links with cognitive performance and behaviour have often not been investigated. A further complicating matter for the interpretation of studies on *SLC6A3/DAT1*

is the switch in ADHD risk allele from childhood to adulthood. Longitudinal studies or at least cross-sectional studies including large samples of both children and adult participants (preferably also including persistent and remitted forms of ADHD) are therefore needed to advance the field.

Findings for the dopamine receptor D4 gene (*DRD4*)

The **dopamine D4 receptor**, encoded by *DRD4*, is a G protein-coupled receptor and belongs to the dopamine D2-like receptor family (Oldenhof et al., 1998). The most widely studied *DRD4* polymorphism in ADHD has been the 48 bp VNTR in exon 3 of the gene, with the 2-, 4-, and 7-repeat alleles being the most common alleles. Allele frequencies vary significantly across ethnic groups (Chang et al., 1996; Van Tol et al., 1992), and the ADHD risk allele in the Caucasian population (7R) seems to be a different one from that in Asians (Li et al., 2006a; Nikolaidis and Gray, 2010; Wang et al., 2004). The *DRD4* 48 bp VNTR seems to show differential association with ADHD in children, where it may be one of the strongest risk factors among the common genetic variants (Gizer et al., 2009), and adults, where no association with the disorder could be observed (Sanchez-Mora et al., 2011), though it might occur as part of gene-environment interactions (Sanchez-Mora et al., 2015). *DRD4* is abundantly expressed in areas of the brain affected in ADHD, including frontal lobe regions, such as the orbitofrontal cortex and anterior cingulate (De La Garza and Madras, 2000; Lahti et al., 1995; Li et al., 2007b); the risk allele has been found to affect receptor binding and to produce a blunted response to dopamine (Asghari et al., 1995), although the functionality of the *DRD4* VNTR has recently been challenged by meta-analysis (Pappa et al., 2015). The exon 3 VNTR has been the sole target of imaging genetic studies involving patients with ADHD, in which different imaging modalities have been used (**Table 3**).

An early sMRI study in children and adolescents did not report differences between carriers and non-carriers of the 7R-allele in selected regional and global brain volumes (Castellanos et al., 1998). A second study observed smaller prefrontal grey matter volumes in children homozygous for the 4R-allele, an effect that appeared particularly pronounced in unaffected siblings of patients with ADHD (Durston et al., 2005). In contrast, adult patients carrying the 7R-allele were found to have smaller volumes of the superior frontal and cerebellar cortex (Monuteaux et al., 2008). In a longitudinal study, *DRD4* genotype also affected cortical development (by measuring cortical thickness), with 7R-carriers showing thinner prefrontal and parietal cortex; patients with ADHD carrying this allele had a distinct trajectory of cortical development characterized by normalization of parietal cortical regions (Shaw et al., 2007b). Additionally, an observational cohort study evaluating the effects of cumulative stimulant treatment, revealed associations between treatment and frontal and hippocampal volume dependent on *DRD4* genotype and age (Schweren et al., 2016). More specifically, carriers of the 7R-allele showed decreased frontal cortex volume at younger age and lower treatment levels, whereas left hippocampal volume was increased

in those with treatment at younger age (Schweren et al., 2016) (**Table 3**). By studying GxE interaction effects, carriers of the 7R-allele were found to have larger putamen volumes over age when exposed to high positive peer affiliation (Richards et al., 2016). This result was independent of ADHD severity. Effects of *DRD4* genotype on structural brain connectivity were investigated in a single study in Asian children with ADHD, resulting in a report of no effects for 4R-homozygotes (Hong et al., 2014). A very large recent study in healthy participants (n=765) revealed an absence of significant effects of the *DRD4* 5R-allele on FA as well (Takeuchi et al., 2015). However, widespread changes in another measure of structural connectivity, mean diffusivity (MD), were observed, with increased MD in 5R-carriers in grey and white matter areas of the cerebral cortex, and in subcortical areas including the globus pallidus, amygdala, midbrain areas, and the brain stem (Takeuchi et al., 2015).

An EEG study administering a CPT as a measure of response inhibition and sustained attention, reported that children with the 7R-allele showed increased frontal θ and reduced global β power. Similar effects of *DRD4* genotype were also apparent on the β frequency band in the parents (Loo et al., 2010); in both generations, carriers of the 7R-allele had reduced cortical activation upon performing the CPT task compared to participants not carrying this allele (Loo et al., 2010). Similarly, Albrecht and colleagues also applied the CPT to a childhood case-control sample and reported significant effects of the 7R-allele on ERP components. Specifically, they observed reduced activity related to attentional orienting and cognitive or response preparation in 7R-carriers (2014). This effect was found to be independent of an ADHD diagnosis (Albrecht et al., 2014). Extrapolating to behaviour, a recent meta-analysis showed that longer *DRD4* variants (including the 7R-allele) were associated with lower levels of executive functioning (Pappa et al., 2015). In contrast to the continuous performance measure, an EEG study of inhibition applying another Go/No-Go task to adults with and without ADHD did not find an effect of *DRD4* genotype on the NGA neural correlate of prefrontal response control (Heinzel et al., 2013). However, in this study, an epistatic interaction between the *DRD4* VNTR and a SNP in the *COMT* gene (rs4680; Val/Met) was observed for the NGA; in homozygous carriers of the *DRD4* 7R-allele, the NGA followed a more pronounced U-relationship based on an increasing number of *COMT* Met-alleles compared with 7R-heterozygotes. In *COMT* Val/Met carriers, *DRD4* 7R-homozygosity was associated with decreased NGA compared with 7R-heterozygosity. This interaction could be localized to the right premotor and supplementary motor areas (Heinzel et al., 2013). These findings were independent of diagnostic status.

Thus, though existing evidence does not support firm conclusions about the pathways from gene to disease, genotypic variability in *DRD4* may affect brain structure and/or activity and mark a particular developmental trajectory in cortical brain structure related to adult outcome of ADHD.

Findings for other ADHD candidate genes

Adrenergic neurotransmitter systems influence attentional processes and certain aspects of executive control (Arnsten, 2006), and the gene encoding the **alpha-2A adrenergic receptor** (*ADRA2A*) has been found to be a candidate gene for ADHD. The alpha-2A adrenergic receptor is the most prevalent noradrenergic receptor in the PFC (Arnsten et al., 1996; Park et al., 2005). Two variants appear to be risk factors for ADHD, the rs1800544 SNP in the promoter region, for which the G-allele was considered risk-increasing (Park et al., 2005), and the rs553668 SNP in the promoter region, for which the T-allele was considered the risk allele (Park et al., 2005). However, meta-analyses have not confirmed associations with ADHD risk for either SNP (Gizer et al., 2009). Alpha-2 adrenoreceptors have also been implicated in other major neuropsychiatric diseases that are often comorbid with ADHD, such as major depressive disorder and schizophrenia (Langer, 2015).

Genotypic effects of *ADRA2A* on the brain were studied in only two studies of Asian children with ADHD. A SPECT study showed that carriers of the non-risk allele for rs1800544 exhibited reduced perfusion in bilateral orbitofrontal regions (Kim et al., 2010). A DTI study investigated the effect of both SNPs (rs1800544 and rs553668) on white matter integrity. Carriers of the rs553668 non-risk-allele (C-allele) showed reduced fractional anisotropy (FA) in the right postcentral gyrus, whereas carriers of the rs553668 T-allele (non-risk group) showed reduced FA in the right middle frontal cortex (Park et al., 2013).

The **Catechol-*O*-methyl-transferase** enzyme (encoded by the *COMT* gene, located at 22q11.21) is involved in the degradation of the catecholamines dopamine and norepinephrine. It is highly expressed in the frontal lobe regions, where it is responsible for the regulation of dopamine levels (Hong et al., 1998). Studies investigating the association between *COMT* and ADHD have largely focused on a functional SNP (rs4860) in exon 4 that leads to an amino acid substitution (valine à methionine). This polymorphism has been shown to considerably affect COMT enzyme activity, such that homozygous carriers of the valine-allele show 3-4 times higher activity than homozygous carriers of the methionine-allele, resulting in decreased dopamine availability in valine homozygotes (Chen et al., 2004). An initial small study suggested that the valine-allele was associated with increased risk for ADHD (Eisenberg et al., 1999), although a recent meta-analysis failed to confirm this association (Lee and Song, 2015). The functional *COMT* rs4680 variant also seems relevant to other psychiatric and cognitive phenotypes. A recent study evaluating the association of the *COMT* genotype with schizophrenia in a systematic review and meta-analysis in 32,816 subjects, revealing a significant association (Gonzalez-Castro et al., 2016). Another recent meta-analysis revealed an association of the *COMT* genotype and reward learning, suggesting that variability in dopamine signalling associated with *COMT* rs4680 influences individual differences in reward processing, which may potentially contribute

to psychopathology characterized by reward dysfunction, such as ADHD (Corral-Frias et al., 2016).

Two very small sMRI studies, both using VBM, examined the genotypic effect of rs4680 on the brain in children with and without ADHD (**Table 3**). Villemonteix and colleagues observed that children with ADHD and at least one methionine-allele had reduced grey matter volume in the insula/IFG relative to children without ADHD (2015). In an additional ROI analysis within the small ADHD group only (n=34), the study showed that those children with ADHD who were homozygous for the valine-allele had increased grey matter volume in the caudate nucleus compared to ADHD children carrying the methionine-allele, and also compared to children without ADHD (Villemonteix et al., 2015). A second sMRI study in a Japanese childhood sample also found a relation of *COMT* genotype with striatal volume pointing in the same direction, as the authors showed that the smaller grey matter volume observed in the left putamen in children with ADHD was moderated by the methionine-allele, whose carriers showed smaller volume than the valine homozygotes (Shimada et al., 2015). Effects of *COMT* on structural connectivity were investigated by two DTI studies. One study in Asian children with ADHD revealed that carriers of the methionine-allele showed a weakened network of white matter connections linking 18 different brain regions (Hong et al., 2014) (**Table 3**). This finding is in line with a report on healthy participants, showing that the methionine-allele was associated with impaired structural maturation of brain white matter connectivity (Thomason et al., 2010). Based on these findings, Hong and coworkers formulated the hypothesis that higher dopamine availability may inhibit myelination (2014). Recently, Kabukcu Basay and colleagues reported that children with ADHD, who were homozygous for the Valine-allele, had reduced FA and increased RD values in the right cingulum bundle (2016). This indicates demyelination effects in the white matter connections between the cingulated cortex to the PFC, premotor regions, cortical association areas in the parietal and occipital lobes, thalamus, and hippocampus (Kabukcu Basay et al., 2016). The cingulated cortex is known to be involved in complex cognitive processing (Bush et al., 2000) and functions that are believed to be impaired in ADHD (Bush, 2011; Makris et al., 2008).

A single fMRI study investigated the association of *COMT* rs4680 genotype on task performance and whole-brain neural activation during response inhibition (van Rooij et al., 2015b). Although the *COMT* Val158Met variant resulted in differential activation patterns during successful and failed stop-trials in the combined ADHD-control sample, no interactions between genetic effects and ADHD diagnostic status were observed in any of the whole-brain fMRI results (van Rooij et al., 2015b) (**Table 3**). This indicates that genetic variation in the COMT gene exerts large-scale effects on neural activation changes of the response inhibition network, but these changes are independent of ADHD.

The single EEG study of *COMT* in ADHD, using a Go/No-Go task in adults with and without ADHD, did not reveal an effect of *COMT* genotype on brain activity individually. However,

an epistatic interaction of *DRD4* x *COMT* genotype on neurophysiological correlates of prefrontal function was observed (Heinzel et al., 2013). In homozygous carriers of the *DRD4* 7R allele, the anteriorization of the NoGo response (NGA, explained above) followed a more pronounced U-relationship with increasing numbers of Met alleles compared with 7R heterozygotes. In *COMT* Val/Met carriers, *DRD4* 7R homozygotes showed a significantly decreased NGA compared with 7R heterozygotes. The genotype-dependent effects on the NGA were localized in the right premotor and supplementary motor area. The epistatic interactions were independent of ADHD diagnosis (Heinzel et al., 2013). Given the role of fronto-striatal circuits and dopamine in reward processing (Plichta and Scheres, 2014; von Rhein et al., 2015), an important domain of impairment in ADHD (see above), it is surprising that no studies have yet investigated the role of *COMT* in brain activity related to reward anticipation and receipt.

The **dopamine D1 receptor** (encoded by the *DRD1* gene) is the most abundant dopamine receptor subtype in the brain; it is highly expressed in the striatum and cerebral cortex (Bergson et al., 1995). Common genetic variation in the *DRD1* gene (rs5326) has been associated with schizophrenia risk (Pan et al., 2014) and impaired cognition in patients with bipolar disorder (Zhao et al., 2015), both often found comorbid with ADHD. Several studies explored associations between ADHD and genetic variants of *DRD1*, such as SNPs rs4532 and rs265981 (Bobb et al., 2005). In the initial study, participants with ADHD were more likely than healthy controls to have the C-allele or rs4532 and the A-allele of rs265981 (Bobb et al., 2005). However, results of a meta-analysis of rs4532 did not support its association with ADHD (Wu et al., 2012), and also replication for rs265981 is still pending. Nevertheless, two sMRI studies investigated the effect of genetic variation in *DRD1* on cortical thickness (Shaw et al., 2007b) and on brain volume (Bobb et al., 2005). Neither study found an effect of *DRD1* genotype or group x genotype interactions (**Table 3**).

The **5-Hydroxytryptamine receptor 1B** (encoded by the *HTR1B* gene) is the most widely studied serotonin receptor gene in relation to ADHD. HTR1B is a G protein-coupled receptor that inhibits cyclic AMP formation (Murphy et al., 1998). It is highly expressed in the dorsal raphe nucleus, which is involved in the sleep/wake cycle, and to lesser degrees in the striatum and frontal regions, such as the dorsolateral PFC (Ichikawa et al., 2005). The initial study, investigating 273 nuclear families with ADHD, reported preferential transmission of the rs6296 G-allele to ADHD probands (Hawi et al., 2002). Results of a meta-analysis supported this association between childhood ADHD and rs6296 genotype (Gizer et al., 2009). A single fMRI study investigated the relationship of rs6296 genotype with neural correlates of response inhibition, using a stop-signal task in a childhood sample (van Rooij et al., 2015a). The rs6296 genotype was associated with widespread differential activation during successful and failed stop trials (**Table 3**). However, the direction of these effects

was inconsistent, with both increased and decreased activation for the GG genotype being observed in frontal and posterior nodes, and the differential activation patterns were independent of ADHD diagnosis (van Rooij et al., 2015a).

The **latrophilin 3 gene** (*LPHN3;* official name Adhesion G Protein-Coupled Receptor L3 [*ADGRL3*]) codes for a member of the LPHN subfamily of G-protein-coupled receptors (GPCRs). Subtype 3 is the most brain-specific LPHN (Ichtchenko et al., 1998; Sugita et al., 1998) and is expressed in regions implicated in ADHD, i.e. the caudate nucleus, cerebellum, amygdala, and cerebral cortex (Arcos-Burgos et al., 2010). *LPHN3* was identified as an ADHD risk gene downstream of genetic linkage studies in multicase families from a genetic isolate. In multisite association studies, initially, a haplotype of three SNPs (rs6551665, rs1947274, and rs2345039) was shown to be associated with ADHD (Arcos-Burgos et al., 2010). The association of *LPHN3* genotypes with ADHD has been replicated in children (rs6551665) (Hwang et al., 2015) and adults with ADHD (multiple markers) (Ribases et al., 2011). By use of proton magnetic resonance spectroscopy (^{1}H-MRS), Arcos-Burgos and colleagues showed that individuals carrying the *LPHN3* susceptibility haplotype exhibited a decreased ratio of N-acetylaspartate to creatine (NAA/Cr ratio) in the left lateral and medial thalamus as well as the right striatum, and an increased ratio in inferior–posterior cerebellar vermis; this suggested that the maintenance of neuron viability is altered in those carrying the ADHD risk haplotype (2010). Since the ADHD susceptibility haplotype itself did not cause any significant coding region changes or canonical splice site alterations, it was suggested that non-coding variations may be likely contributors to ADHD (Domene et al., 2011).
Although *LPHN3* is among the best-supported candidate genes for ADHD, thus far, imaging genetics studies of the gene are still limited in the literature. No studies on structural or connectivity alterations related to the risk variant of the gene in patients with ADHD have been published yet, nor have any fMRI studies. An EEG study using a Go/No-Go task revealed that adult patients with ADHD carrying a 'high-risk' *LPHN3* haplotype (comprised out of rs2305339, rs734644, rs1397547, and rs1397548) showed a more anterior Go-centroid of the P300, had a reduced NGA, and had worse behavioural task performance due to more omission errors (Fallgatter et al., 2013).

Monamine oxidase A, encoded by *MAOA*, is an enzyme, which catalyzes the oxidative deamination of amines, such as dopamine, norepinephrine, and serotonin. For this gene located on the X-chromosome, studies have largely focused on a functional 30 bp VNTR 1.2 kb upstream of the gene, which has been previously associated with impulsivity and aggression (Caspi 2002, Manuck 2000). The polymorphism consists of alleles of 2, 3, 3.5, 4, and 5 repeat copies, and evidence suggests that the 2 and 3 repeat ('low-activity/*MAOA*-L') alleles are less efficiently transcribed than the longer ('high-activity/*MAOA*-H') alleles (Deckert et al., 1999). Although the *MAOA* gene has received a lot of attention as a candidate

gene for ADHD given the prior findings for impulsivity, a meta-analysis did not indicate a significant association between ADHD and the high activity alleles of the VNTR (Gizer et al., 2009). A SNP, rs1137070 (located in exon 14), has also been reported to contribute to impulsivity and the outcome of ADHD (Li et al., 2007a; Liu et al., 2011). Especially, the C-allele of rs1137070 has been associated with high ADHD scores and poor outcomes (Li et al., 2007a). With evidence for a role of MAOA in aggression being more consistent than for ADHD (e.g. (Brunner et al., 1993; Byrd and Manuck, 2014; Caspi et al., 2002), imaging genetics studies have largely concentrated on population groups other than patients with ADHD, e.g. (Holz et al., 2016; Meyer-Lindenberg et al., 2006). A recent fMRI study forms an exception. Using a phonological working memory task in male Asian adults with and without ADHD, the authors showed that the effect of *MAOA* (rs1137070 T- versus C-allele) interacted with diagnosis in the left inferior frontal lobe, pars opercularis (**Table 3**); further analysis demonstrated that the increased brain activation observed in this region in patients was only significantly different to controls among those hemizygous for the T-allele (Ko et al., 2015).

The *NOS1* gene codes for **nitric oxide synthase 1**, an enzyme that synthesizes nitric oxide from L-arginine. Nitric oxide is a reactive free radical, which can act as a biological mediator in several processes, including dopaminergic and serotonergic neurotransmission (Kiss and Vizi, 2001) and neurite outgrowth (Chen et al., 2006). The *NOS1* gene has a complex structure, including 12 alternative untranslated first exons (exon 1a-1l). In exon 1f, a VNTR that affects gene expression has been linked to hyperactive and impulsive behaviour in humans (Reif et al., 2009; Weber et al., 2015); the short allele was shown to be the risk factor for ADHD, especially in females.

No studies have yet reported effects of *NOS1* on brain structure. A recent case-control DTI study of structural connectivity in adolescents revealed that female homozygous carriers of the ADHD risk allele showed higher MD values in several major white matter tracts of the brain compared with long allele carriers. This effect was present in both female patients and controls (Van Ewijk et al., in revision). The white matter tracts found affected by *NOS1* genotype overlap with those earlier found associated with ADHD (Onnink et al., 2015; van Ewijk et al., 2012; Wu et al., 2016). Since higher MD values can be indicative of demyelination, lower axonal density, or axonal degeneration, homozygosity of the short allele might thus be a risk factor for aberrant development of white matter tracts involved in ADHD etiology.

NOS1 exon 1f is particularly highly expressed in striatum. Therefore, the single study of gene effects on brain function investigated the effect of the VNTR on ventral striatal activity during reward anticipation using fMRI (Hoogman et al., 2011) (**Table 3**). The study revealed that homozygous carriers of short alleles of *NOS1* demonstrated higher ventral striatal activity than carriers of the other *NOS1* VNTR genotypes (Hoogman et al., 2011). Again, this effect was independent of diagnostic status. Similar effects of the genotype were also observed for behavioural impulsivity, with those carrying the ADHD risk factor acting

more impulsive than other participants. As the authors did not perform mediation studies, it remains to be investigated, whether the observed genotype effects on brain connectivity and/or activity directly link to behavioural effects.

The **norepinephrine transporter** gene (*SLC6A2*, *NET1*) codes for a protein responsible for the reuptake of norepinephrine (as well as dopamine) from the synaptic cleft back into the presynaptic neuron (Pacholczyck 1991). The gene is highly expressed in the frontal lobes (Stahl, 2003). Candidate gene studies of *SLC6A2*/*NET1* selected numerous SNPs to test for association, but conflicting results have been reported, with each study yielding evidence of association, but differing in which specific SNPs were associated with ADHD (Gizer et al., 2009).

A SPECT study examined the effects of rs5569 and rs28386840 on cerebral perfusion in response to MPH treatment in Asian children with ADHD (**Table 3**) (Park et al., 2012). At baseline, no differences were observed, but after eight weeks of MPH treatment increased regional brain perfusion in the right inferior temporal gyrus and middle temporal gyrus in homozygous carriers of the rs5569 G-allele was demonstrated (Park et al., 2012). Given that no previous studies had reported a significant association between this polymorphism and ADHD, no 'risk' allele was indicated. A PET study reported the influence of four genetic variants within the transporter gene on *in vivo* norepinephrine transporter binding in adults with and without ADHD (**Table 3**) (Sigurdardottir et al., 2015); the authors found differences in cerebellar and thalamic norepinephrine transporter binding depending on genotype between adult patients and controls (Sigurdardottir et al., 2015). For the two SNPs rs28386840 and rs2242446, patients carrying the major alleles (A/T) showed increased norepinephrine transporter binding in the thalamus compared to controls carrying the major alleles. For the SNPs rs15534 and rs40615, controls carrying the major alleles (C/T) showed increased norepinephrine transporter binding in the cerebellum compared to patients carrying the major alleles (Sigurdardottir et al., 2015). In the patients with ADHD, a high correlation between hyperactivity/impulsivity symptoms and norepinephrine transporter binding in the cerebellum was detected, an effect which was strongly moderated by genotype (Sigurdardottir et al., 2015).

With this knowledge on SNP functionality related to ADHD, studies using additional imaging modalities are warranted, but thus far, only a single sMRI study investigated the effect of genetic variation in *SLC6A2*/*NET1* on brain volume (**Table 3**). No effect of *SLC6A2*/*NET1* genotype (for SNPs rs998424 and rs3785157 – different SNPs from those having been investigated through PET and SPECT) and no group x genotype interactions were reported (Bobb et al., 2005).

The **serotonin transporter** gene (*5HTT, SERT;* official name *SLC6A4*) codes for a solute carrier protein responsible for the reuptake of serotonin from the synaptic cleft back into

the presynaptic neuron, which is the primary mechanism for regulating serotonergic activity in the brain (Lesch et al., 1996). A functional polymorphism exists in the promoter region of this gene (5HTTLPR) in the form of a 44-bp insertion/deletion yielding short (S) and long (L) alleles. The long variant is associated with more rapid serotonin reuptake, resulting in lower levels of active serotonin (Lesch et al., 1996). A SNP in the long allele, rs25531, additionally modifies its activity (Lesch et al., 1996). *SLC6A4/5HTT* has been implicated in emotion regulation, (emotional) memory, and learning processes (Araragi and Lesch, 2013; Barzman et al., 2015; Meneses and Liy-Salmeron, 2012). The serotonin transporter is expressed in regions implicated in attention, memory, and motor activities, such as the amygdala, hippocampus, thalamus, putamen, and ACC (Frankle et al., 2004; Oquendo et al., 2007). The 5HTTLPR has been extensively studied for its role in depression and anxiety – especially in the context of environmental adversity, with the S-allele being the risk allele (Caspi et al., 2003; Oo et al., 2016). For ADHD, the evidence for association is more limited, although an earlier meta-analysis provided significant evidence of an association between ADHD (in children) and the 'long' variant of 5HTTLPR (Gizer et al., 2009). However, an international multicentre study reported a slight, non-significant overrepresentation of the S-allele in adult patients with ADHD (Landaas et al., 2010).

Given the evidence for *SLC6A4* as a depression gene, imaging genetics research has largely concentrated on non-ADHD samples; in healthy individuals, functional genetic variation in the *SLC6A4/5HTT* gene has been linked to emotion regulation through effects on brain activation in the amygdala and the wider 'threat circuit', while effects on regional brain volumes are inconsistent (Jonassen and Landro, 2014; Klein et al., under review). Three sMRI studies have been performed for 5HTTLPR in ADHD thus far, all investigating GxE interactions. The initial study showed that the interaction between exposure to environmental stress and carriership of the 5HTTLPR S-allele, which was linked to increased ADHD severity in a longitudinal study of ADHD families (van der Meer et al., 2014), was associated with reduced cortical grey matter volume in the precentral gyrus, middle and superior frontal gyri, frontal pole, and cingulate gyrus in S-allele carriers compared with participants homozygous for the L-allele (van der Meer et al., 2015a). Importantly, this paper showed that only some of these regions, the frontal pole and the ACC, actually mediated the effects of the gene-environment interaction on ADHD severity. Similarly, van der Meer and colleagues reported that individuals carrying the *NR3C1* risk haplotype, who were homozygous for the 5HTTLPR L-allele, showed a negative relation between stress and grey matter volume (2016). However, no mediation effects were found, meaning that the local effects of these interaction on grey matter volume did not significantly explain their association with ADHD severity (van der Meer et al., 2016). Such studies testing the mediation of effects through the observed brain phenotypes (as opposed to those just being epiphenomena of the genetic variation) are largely lacking in the imaging genetics literature thus far, but are critically needed to map gene to disease pathways. However,

sample sizes will have to be substantial to allow valid conclusions to be drawn from such studies. Another sMRI study used the same dataset and revealed that, in agreement with age-related reductions of total grey matter volume found in longitudinal studies (Brain Development Cooperative, 2012; Raznahan et al., 2014), participants scoring high on positive peer affiliation and carrying two 5HTTLPR L-alleles had smaller total grey matter volumes with age (Richards et al., 2016). Moreover, participants with the same genotype, but low positive peer affiliation had larger GM volumes with age (Richards et al., 2016). These findings were independent of ADHD severity and were in line with a longitudinal study reporting regional GM reductions with age in adolescents exposed to high positive maternal behavior, but increased putamen volumes when exposed to maternal aggression (Whittle et al., 2014).

In contrast to the sMRI studies, which investigated GxE effects related to stress and social environments, the only fMRI study focused on the relationship between 5HTTLPR genetic variation and neural correlates of response inhibition using a stop-signal task (van Rooij et al., 2015a) (**Table 3**). Using a childhood sample, van Rooij and colleagues revealed that homozygous carriers of the 5HTTLPR S-allele showed decreased activation in the frontal nodes and increased activation in the posterior nodes in successful stop trials (2015a). However, no significant associations were found between differential neural activation and ADHD diagnosis or ADHD severity (van Rooij et al., 2015a).

The **synaptosomal-associated protein 25** (encoded by the *SNAP25* gene) is involved in axonal growth and synaptic plasticity, as well as in the docking and fusion of synaptic vesicles in presynaptic neurons necessary for the regulation of neurotransmitter release (Sollner et al., 1993). Several studies have tested for linkage and association between *SNAP25* and ADHD, and these studies have consistently genotyped multiple SNPs within the gene rather than focusing on any single polymorphism. An early meta-analysis of SNP rs3746544 suggested *SNAP25* as ADHD risk gene (Faraone et al., 2005). A more recent meta-analysis of rs3746544 also provided evidence of a modest but significant association between childhood ADHD and the T-allele, whereas no evidence of association with rs1051312 was found (Gizer et al., 2009).

Again, only a single imaging genetics study is currently available for this gene. In this case, the interaction of MPH treatment-related hemodynamic changes with genetic variation in *SNAP25* (rs3746544 and rs1051312) was studied in small samples of children and adults with ADHD on and off single dose MPH using fNIRS (**Table 3**). Through fNIRS, brain haemodynamics in prefrontal cortices can be measured, since brain activation causes increased cerebral blood flow, but not all of this oxygenized blood is used, therefore oxygenated haemoglobin increases and deoxyhaemoglobin decreases during e.g. sustained attention (Villringer and Chance, 1997). Homozygous carriers of the rs3746544 T-allele exhibited changes in right oxygenated haemoglobin and right as well as left deoxyhaemoglobin levels. Additionally, homozygous carriers of the rs1051312 T-allele

showed decreased right prefrontal deoxyhaemoglobin with treatment. Combination of the genotypes also showed interaction effects on right prefrontal deoxyhaemoglobin levels (Oner et al., 2011).

Tryptophan hydroxylase 2 catalyzes the reaction of tryptophan to 5-hydroxytryptophan, which is subsequently decarboxylated to form the neurotransmitter serotonin. Two isoforms of tryptophan hydroxylase have been identified (encoded by the *TPH1* and *TPH2* genes). The *TPH2* gene codes for a rate-limiting enzyme in the production of serotonin in serotonergic neurons in the midbrain raphe nuclei, while *TPH1* seems to be involved in synthesizing serotonin in peripheral tissues (Walther et al., 2003). For several SNPs of the *TPH2* gene, an association with ADHD was found (Brookes et al., 2006a; Sheehan et al., 2005; Walitza et al., 2005). For rs4570625, the G-allele was shown to be transmitted more often to offspring with ADHD (Walitza et al., 2005). For rs11178997, the T-allele was identified as the risk allele for ADHD (Walitza et al., 2005). However, the initial findings could not be replicated in a larger, multicentre study (Brookes et al., 2006a). Mouse models in conjunction with approaches focusing on *TPH2* variants in humans described a role of serotonin in brain development and in disorders related to negative emotionality, aggression, antisocial behaviour, and bipolar disorder (Gao et al., 2016; Gao et al., 2012; Waider et al., 2011). The *TPH2* gene has also been a candidate for the investigation of gene by environment interaction studies, in depression and suicidal behaviour as well as in aggression; for review see (Lesch et al., 2012; Mandelli and Serretti, 2013).

As the evidence for a role of *TPH2* in ADHD is limited, only a single imaging genetics study involving patients with this disorder has yet been performed for this gene. This EEG study investigated the genotypic effect of the two SNPs of the *TPH2* gene mentioned above (rs4570625 and rs11178997) on the NGA during a Go/NoGo task in adult patients with ADHD and healthy individuals (**Table 3**). Risk alleles of each of the SNPs were found associated with a reduction in the NGA in both participant groups, indicating an effect on ADHD-relevant prefrontal brain function independent of a specific psychiatric diagnosis (Baehne et al., 2009). These promising first findings may warrant further analysis of *TPH2* variants, especially in combination with adverse environmental factors.

Outlook

In this review we set out to summarize the current literature on imaging genetics studies of candidate genes involving patients with ADHD. As no genome-wide association studies have yet reported loci/genes with significant evidence for association with ADHD, we used a liberal definition of a candidate (having shown association with the disorder in at least one earlier study) and used an adapted version of the list of ADHD candidate genes

recently compiled by Li and coworkers (2014). Of the 62 candidate genes selected for review, we found that only 12 had been studied with any brain imaging technique in an ADHD population. For most of those 12, only a very limited number of studies was available. The two most frequently studied ADHD candidate genes were the *SLC6A3/DAT1* and *DRD4* genes, and even for those two genes most findings await replication.

Brain imaging phenotypes offer an important level of investigation in mapping the biological pathways from gene to disease. Brain structure, function, and connectivity can provide endophenotypes (or intermediate phenotypes) for a disease (Gottesman and Gould, 2003). As those brain endophenotypes are thought to lie in-between a genetic factor and the clinical phenotype, it has been argued that effect sizes for effects of genes on those brain phenotypes may be larger than the ones for effects on behaviour. However, for structural brain phenotypes (i.e. brain volumes) and for structural connectivity, this has been refuted by recent large-scale studies; see (Franke et al., 2016; Jahanshad et al., in preparation). For brain activity measured by fMRI, comparisons of cognitive and brain imaging studies for several genetic variants (Rose and Donohoe, 2013) and evidence from meta-analyses (Mier et al., 2010; Murphy et al., 2013) suggests that effect sizes might be larger. However, it is still unclear, how large the effects of publication bias on those results are (Murphy et al., 2013). Nevertheless, power estimates indicate that several hundred samples are needed, while the majority of studies discussed in this review had less than 50 participants per group (see **Table 3**). This has implications for the generalizability/replicability of positive findings (as the sample investigated may not sufficiently well represent the population it was sampled from) as well as harbouring the risk of false-negative findings (Button et al., 2013). Importantly, the term endophenotype has also often been misused in recent years, as for many of the so-called endophenotypes, the required criteria have not all been investigated. As such, many of the endophenotypes may not be intermediates between gene and behavioural phenotype, but rather be simple markers of disease (Kendler and Neale, 2010). Assessing, whether a brain phenotype observed for a risk genotype really mediates between the risk variant and the behavioural/clinical phenotype has been done in hardly any of the papers reviewed here. The paper by van der Meer and colleagues, in which such an analysis has been performed, shows the importance of such mediation analyses, as only a subgroup of the brain substrates of the gene-environment interaction investigated was also linked to ADHD severity (2015a).

Shortly summarizing the results of the review, our understanding of the mechanisms underlying the action of genetic risk factors for ADHD is still limited. A promising start has been made. We see, for example, that *SLC6A3/DAT1* genotype regulates dopamine activity in striatal regions implicated in ADHD neurobiology (Spencer et al., 2013). Genetic variants also appear to affect brain structure and function beyond the regions of gene expression (Hong et al., 2014; van der Meer et al., 2014; Villemonteix et al., 2015), which is likely due to effects on structural and/or functional connectivity. Some studies reported differential

genotype effects for ADHD patients and controls, whereas others did not observe genotype by diagnosis interactions (**Table 3**). However, such findings have not been replicated yet, and genotype effects may differ in the context of other ADHD-related risk factors.

As there is room for improvement of study designs, and research focusing on new ADHD candidate genes identified through hypothesis-free, genome-wide association studies will soon be needed, we address main challenges and opportunities for imaging genetics studies in the following paragraph. Additionally, since imaging genetics approaches cannot explain the full complexity of a biological system such as the human brain, we also highlight additional levels of investigation and methodologies that can complement the insights provided by imaging genetics studies.

Main challenges and opportunities for imaging genetics studies

The size of individual studies is potentially the biggest challenge in imaging genetics today, as also discussed above. Given the small (to potentially modest) effect sizes to be expected (Franke et al., 2016; Murphy et al., 2013; Rose and Donohoe, 2013), larger samples are essential in order to gain the necessary statistical power.

Common confounding factors encountered in the published brain imaging genetics studies are mainly related to the study sample itself, and many are aggravated by the limited sample sizes used.

False-negative findings may occur due to the large variability of the ADHD phenotype. Additionally, comorbidities occur frequently in ADHD (Kessler et al., 2006; McGough et al., 2005; Wilens et al., 2009), and differences in medication use may further increase phenotypic heterogeneity (Schweren et al., 2013). Together with the low effect sizes of individual common genetic variants and limited sample sizes, phenotypic heterogeneity in a sample challenges the discovery of effects. Meaningful subtyping of this heterogeneous condition to decrease phenotypic heterogeneity and maximize power might therefore be helpful. Besides that, potential differences of effects between age groups, with gender distribution, intelligence levels, and between ethnicities can still make it challenging to replicate and generalize findings.

Although most genetic risk factors investigated here were derived from studies of ADHD in children, our review shows that imaging genetics studies have employed childhood, adolescent, and adult ADHD samples. Knowing about the age-dependence of the genetic contribution to ADHD (Chang et al., 2013; Pingault et al., 2015), which e.g. results in differential association of *SLC6A3/DAT1* (Franke et al., 2010) and *DRD4* (Sanchez-Mora et al., 2011) with ADHD in children and adults, makes the definition of appropriate research questions highly important. Only one study thus far used a longitudinal approach, with a follow-up period of six years (Shaw et al., 2007b). Longitudinal samples are best suited to investigate potential age-dependent changes in the effects of genetic factors on the brain, and therefore more longitudinal studies of ADHD including imaging and genetic assessments are needed. This

is especially important in light of a recent discussion on whether not only the onset of ADHD can occur in adulthood, but also whether childhood-onset and adult-onset ADHD may be distinct syndromes or trajectories (Agnew-Blais et al., 2016; Caye et al., 2016; Faraone and Biederman, 2016; Moffitt et al., 2015). Therefore, longitudinal studies including participants with remitted versus persistent ADHD, can help us in understanding the genetic and neurobiological correlates of this multifactorial disorder.

All of the imaging genetics studies reviewed here employed hypothesis-driven approaches. This means, that candidate genetic variants were only investigated in relation to specified candidate brain phenotypes and by this it severely limits our ability to create new knowledge on genetic effects on the brain. Even main effects of a variant may have been missed, as our ability to define the right hypotheses is hampered by the still so limited understanding of ADHD etiology. Besides that, most studies investigated single genetic variants (SNPs or VNTRs), although we know that frequently more than one risk allele exists in a single gene/locus; see e.g. (Schizophrenia Working Group of the Psychiatric Genomics et al., 2014). Also, only few studies looked for epistatic or gene-environment interaction effects.

In the face of limited sample sizes available for an imaging genetics study, data reduction strategies might help to preserve power. By moving to gene-based mass-univariate and multivariate statistics, it is possible to test the combined effect of multiple genetics variants in a single test statistic. Such models thus reduce the number of statistical tests, e.g. through gene-wide analyses, and - by explaining more phenotypic variance - may enable the discovery of gene effects that would have been otherwise undetectable with single variant methods (Bralten et al., 2011; Bralten et al., 2013; Hibar et al., 2011). Alternatively – or rather in addition - analyses of multiple genetic factors may be performed in the context of international collaborations, like ENIGMA and CHARGE (Psaty et al., 2009; Thompson et al., 2014) or using the publicly available data from large national research efforts (like the UK Biobank; http://www.ukbiobank.ac.uk/). These are mainly population studies, but since ADHD is currently viewed as an extreme on a continuum of population traits (Chen et al., 2008; Larsson et al., 2013a), we can learn a lot from studies performed in healthy individuals. While for this review, we specifically selected studies that had included patients with ADHD, it is indeed apparent also from those studies that genes affect traits rather than disorders (Hoogman et al., 2011). We have referred to some relevant studies in healthy individuals already in the previous sections. An additional example is an fMRI study investigating a genetic variant in *SNAP25*, which was found associated with altered activation of the posterior cingulate cortex during a working memory task, a finding that was replicated in a second, independent sample (Soderqvist et al., 2010). With working memory being one of the cognitive domains affected in ADHD (Alderson et al., 2013), this finding can contribute to unravelling the biological pathways from gene to disease. However, some studies have also suggested differential genotype effects in subjects with ADHD and healthy participants (Durston et al., 2008; Monuteaux et al., 2008; Onnink et al., 2016). In those cases, imaging

genetics studies in healthy participants will not reveal the full spectrum of the genetic effects, and studies in patients are warranted. The ENIGMA-ADHD Working Group, with its sample spanning 60 years of the human lifespan (Hoogman et al., submitted), is an important resource for the investigation of diagnosis-specific effects.

There are a number of additional areas, in which we foresee that imaging genetics studies will profit from technical advances as well as recent insights into disease etiology. Importantly, a new GWAS meta-analysis is now underway by the ADHD Working Group of the Psychiatric Genomics Consortium (PGC) and the Danish iPSYCH consortium, which will provide several bona fide ADHD risk genes identified in hypothesis-free analyses (PGC and iPSYCH ADHD working groups, in preparation). Those will be interesting targets for investigation through imaging genetics approaches. In addition, the introduction of next generation sequencing (NGS) technology in psychiatric research will allow us to go beyond studying SNPs and VNTRs. It is likely that also more rare genetic variants, identified through exome and whole-genome sequencing will find their way into imaging genetics studies, e.g. through mutational load or burden tests (Medland et al., 2014).

Most imaging genetics studies in the field of ADHD have used MRI as their main brain imaging technique. For subcortical brain structures, grey matter volume has been the most frequently investigated characteristic. For the cortex, measurements of surface area (SA) and cortical thickness (CT) were found to be genetically and phenotypically rather independent (Winkler et al., 2010). Volume has been shown to be more closely related to SA than CT (Winkler et al., 2010). Therefore, it has been suggested that SA and CT measurements should be considered separately in imaging genetics studies (Winkler et al., 2010), in addition or instead of cortical volume. Until now, only very few studies have made use of this opportunity, however. Because of its low invasiveness, high spatial resolution, and wide availability of MRI scanners, this technique dominates the brain imaging field. Imaging genetic studies investigating genetic variation in the *SLC6A2/NET1, SLC6A3/DAT1,* and *DRD4* also used PET and SPECT (**Table 3**). These modalities enable direct localization and quantification of e.g. binding capacities of transporters and receptors, but are quite invasive. An alternative method for investigating the neurochemistry of the brain *in vivo* might be proton magnetic resonance spectroscopy (^{1}H-MRS), which allows for non-invasive quantification of several neurometabolites, such as N-acetylaspartate, (phospho-) creatine, choline, myo-inositol, glutamate and glutamine, and gamma aminobutyric acid (GABA) (Naaijen et al., 2015). While no significant differences in GABA levels were found in ADHD compared with controls (Schur et al., 2016), a possible increased signal of a combination of glutamate, glutamine, and GABA in the striatum of ADHD patients was observed, as well as an increased signal in the ACC in a paediatric ADHD sample and a reduced signal in an adult ADHD sample; reviewed by (Naaijen et al., 2015). The neurodevelopmental changes in fronto-striatal glutamatergic circuits across the lifespan suggested by this might be interesting targets for future imaging genetics studies. By combining different

methods, it should be possible to create a comprehensive picture of how polymorphisms in ADHD-related genes affect the brain at chemical, structural, and functional levels. To date, also no MEG genetics studies has been reported. The high temporal resolution of this modality might be of great additional value in understanding genetic effects on brain function. Resting-state fMRI studies are also still lacking from the imaging genetics literature. Especially the combination and integration of different modalities in the study of individual participants may provide more comprehensive insights into gene effects (Kobiella et al., 2011). With respect to functional brain markers that might serve as a useful endophenotype, it is crucial to use a functional contrast that isolates brain activity specifically associated with the cognitive process of interest. For example, stop/NoGo and Go conditions in traditional stop-signal/Go-NoGo tasks not only differ in the involvement of response inhibition, but also in other processes such as novelty/probability of occurrence and perceptual processing (Boehler et al., 2010; Sanchez-Carmona et al., 2016). Results of a recent study highlight the importance of controlling for the different strategies adopted by participants to perform selective stopping tasks before analyzing brain activation patterns (Sanchez-Carmona et al., 2016). Thus, activity emerging from a functional contrast (e.g., Go versus NoGo) will probably reflect a mixture of different processes. This is an important issue to be considered, when interpreting task-based fMRI studies and designing new studies.

Another opportunity to improve the design of imaging genetics studies is the use of mediation and moderation analyses. In mediation analysis, a causal explanation for the effect of an independent on a dependent variable is statistically modelled. The assumption of causality is important in this type of analysis and thus should be justified by a plausible biological theory or appropriate experimental constraints. In moderation analysis, the influence of a third variable on the association of an independent and dependent variable is modelled. The simple moderation and mediation models can be combined to account for more complex data structures and biological models (Hayes and Scharkow, 2013; van der Meer et al., 2014). Such models have e.g. been employed in psychological (Aram et al., 2010; Graziano et al., 2011) and medical research questions (Nigg et al., 2008), but could be also promising for analyses of the pathway from genes to phenotypes in complex disorders, such as ADHD, which involves different biological and non-biological factors acting synergistically during an individual's developmental trajectory (Bale et al., 2010; Krain and Castellanos, 2006). Indeed, van der Meer and coworkers recently used such analyses to inspect the moderation of the effect of stress on ADHD severity by the 5HTTLPR genetic variant. The researchers could show that an interaction of 5HTTLPR genotype and stress was associated with ADHD severity (van der Meer et al., 2014), and that this gene-environment interaction had several substrates in the brain. Importantly however, only a subset of those substrates did really mediate the effects of the gene-environment interaction, whereas others were epiphenomena (van der Meer et al., 2015a).

While imaging genetics studies with the goal of mapping pathways from gene to disease up to now have always started with the selection of the candidate gene/variant to study, the advances brought about by international consortia now start to allow entirely data-driven approaches to be used for imaging genetics. For example, we have recently developed a comprehensive pipeline for the analysis of genetic overlap of GWAS results for disease risk (in that case schizophrenia risk data from the PGC) and the GWAS results for brain volume (Franke et al., 2016). The latter was based on data from the ENIGMA consortium (Medland et al., 2014; Stein et al., 2012; Thompson et al., 2014), in which MRI scans and genome-wide genotyping data from up to 30,717 individuals were analysed, and several genetic factors contributing to the volumes of specific brain structures were identified (Hibar et al., 2015). Finding such overlap between disease risk variants and those for brain phenotypes would be indicative of etiologic sharing, and would thus directly flag the pathways from gene to disease (Franke et al., 2016). For their previous studies, ENIGMA used mainly regional brain volume measures (Hibar et al., 2015; Stein et al., 2012), but one can also envisage more comprehensive voxel-wise genome-wide scans (vGWAS) to examine evidence for associations across the genome at each voxel in the brain image (Stein et al., 2010). While these approaches still are limited by the fact that a stringent correction for type I errors dramatically increases the threshold for statistical significance (as genomes and images are both highly dimensional), data reduction strategies are being devised that can preserve power in such settings (Medland et al., 2014). With respect to the different imaging modalities, structural MRI and DTI seem to be best suited for larger collaborations and data sharing, but also fMRI imaging genetics meta-analyses have been shown to be feasible (Mier et al., 2010; Murphy et al., 2013). First analyses of overlap between ADHD GWAS findings and results of the ENIGMA volume analyses are currently underway (Klein et al., in preparation).

Complementary methods for the evaluation of the mechanisms underlying ADHD risk genes

Imaging genetics analyses of the human brain provide information on the effect of ADHD risk genes/variants on brain structure, activity, and connectivity, but other levels of investigation are needed to provide a more complete picture (**Figure 2**). We also need information about the molecular networks, in which an ADHD gene acts, and the cellular processes that are affected by it. In the following section we highlight different methodologies and approaches that can be used to shed light on these additional levels of complexity contributing to the mechanisms underlying the effects of ADHD genes on behaviour and disease.

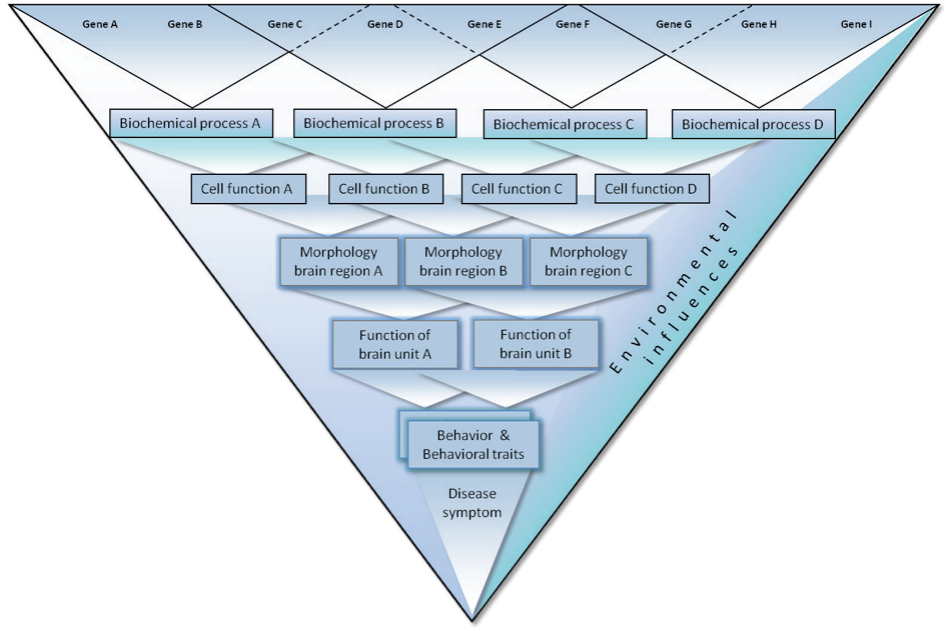

Figure 2: This schematic representation of the endophenotype concept shows the pathway from gene to disease at different levels of complexity in psychiatric genetics. The figure has been modified from a previous publication (Figure 1, (Franke et al., 2009)). Polygenicity (schematically depicted by gene A to I) is involved in causing disease symptoms. A reduced number of genes is involved in disease-related endophenotypes. These can be studied at various biological levels, e.g. biochemical processes and cell function can be assessed by biological assays in cell or animal models by measuring e.g. neuron morphology or synaptic functioning. Neuroimaging methods (structural and functional) can be applied to assess relevant endophenotypes at the level of brain morphology ('Morphology brain region A-C'). Endophenotypes, related to the 'function of brain units', can be e.g. investigated by functional MRI or through performance measurements on neuropsychological tests. Aberrations at this level can result in altered behavior and disease-related behavioral traits, that subsequently lead to disease symptoms. Environmental influences can impact on all levels and need more attention in future studies. Bioinformatic pathway and network analyses can help to integrate data from various sources and to identify molecular networks or cellular processes in which ADHD-related genes are enriched.

Bioinformatics approaches help to integrate findings from different types of molecular studies

Bioinformatics is a broad field of research, which can support our efforts to map pathways from gene to disease in several different ways. A first goal to be pursued through bioinformatics analyses is the clarification of the actual effects of risk variants on gene expression and regulation. Risk variants for a disease are often found in non-protein coding sequences, and therefore the molecular consequences are difficult to evaluate (Civelek and Lusis, 2014; Paul et al., 2014). We will not go into this type of bioinformatics analyses any deeper, but would want to point the interested reader to a recent example for a comprehensive bioinformatic follow-up study demonstrating how to elucidate

the mechanisms by which a genetic risk variant (e.g. rs1344706; *ZNF408A* gene) confers susceptibility to disease, in this case schizophrenia and bipolar disorder (Hess et al., 2015).

Bioinformatics can also help to unravel the molecular networks and cellular processes that an ADHD risk gene is involved in. ADHD-associated genetic factors are distributed throughout the genome; however, they have been found to be enriched within functional categories. This clustering of ADHD-related genes within functional networks or pathways has helped to identify biological processes of importance to ADHD etiology. As also mentioned earlier, through those bioinformatics studies we learned that functions related to nervous system development, cell migration, neuron projection morphogenesis/neurite outgrowth, oxogenesis, cell-cell communication, glutamatergic synapse/receptor signalling, multicellular organismal development, RhoA signalling, glycosaminoglycan biosynthesis, fibroblast growth factor receptor activity, ion (potassium) channel function, transmembrane transport, as well as synaptic transmission, catecholamine metabolic processes, G-protein signalling and organonitrogen compound catabolic processes are enriched in the results of hypothesis-free, genome-wide ADHD-GWAS and CNV studies (Cristino et al., 2014; Hawi et al., 2015; Mooney et al., 2016; Poelmans et al., 2011; Thapar et al., 2015; Yang et al., 2013). Those studies mainly used childhood ADHD data; little is yet known about biological pathways leading to persistence of ADHD. A first, small-scale GWAS of rare and common genetic variants in adults with persistent ADHD showed that the top SNPs implicated biological pathways involved in the regulation of gene expression, cell adhesion, and inflammation (Zayats et al., 2015). The Network and Pathway Analysis Subgroup of the PGC focussed on common pathways underlying additional, ADHD-related psychiatric disorders in adults, i.e. schizophrenia, major depression, and bipolar disorder (The Network Pathway Analysis Subgroup of the Psychiatric Genomics Consortium, 2015). Histone methylation processes (playing important roles in the regulation of gene expression) showed the strongest association, and the researchers also found significant evidence for involvement of immune and neuronal signalling pathways and for processes occurring at the postsynaptic density (The Network Pathway Analysis Subgroup of the Psychiatric Genomics Consortium, 2015).

Comprehensive studies applied to the ADHD-related autism spectrum disorders (ASD) point out, how the combination and integration across several molecular modalities can advance our knowledge about pathways from gene to disease. For example, systematic integration of findings from multiple levels of genomics data and studies of mouse models highlighted the period of fetal development and the processes of chromatin structure, neurite outgrowth, steroidogenesis, synaptic function, and neuron-glial signalling (Chen et al., 2015; Poelmans et al., 2013). Additional studies revealed that ASD genes grouped together in terms of functional annotations, protein-protein interactions and coexpression (Ben-David and Shifman, 2012; Neale et al., 2012; O'Roak et al., 2012; Voineagu et al., 2011), and gene interaction and coevolutionary patterns (Gilman et al., 2011). By integrating gene expression data representing normal human fetal development, developmental timing and

cellular specificity of the molecular pathways disrupted in ASD could be clarified (Parikshak et al., 2013; Willsey et al., 2013). Such integrative studies are still lacking for ADHD, but highly warranted in order to shed more light on the onset and neurodevelopmental trajectory of the disorder.

Animal models provide proof of causality for genes and molecular processes found associated with ADHD

The complexity and crucial role of the brain in the human body largely restrict studies in humans to the described non-invasive imaging methods for investigating brain structure and function. However, to fully understand the role of a disease gene in the fine-tuning of this sophisticated organ, we also need to be able to manipulate genes and monitor the effects of such manipulation on molecular and cellular processes. For this, animal models are indispensable. They can be genetically modified to enable determination of causality, have a natural complexity of the nervous system, and allow a tight control of environmental influences, including diet and drug delivery (Lange et al., 2012; van der Voet et al., 2016). During the last decades, studies were performed on several ADHD animal models, including monkey (Ma et al., 2005; Seu et al., 2009), rat (Ruocco et al., 2014; Russell et al., 1995; Williams et al., 2009b), mouse (Wallis et al., 2012; Zhu et al., 2014; Zimmermann et al., 2015), zebrafish (Lange et al., 2012), and fruit fly (van der Voet et al., 2016). The particularity of each model, like the different levels of complexity of the nervous system, provide complementary information to the broad picture of ADHD.

Most studies in monkeys were mainly based on drug administration to stimulate/inhibit certain brain regions or neurotransmitter receptors and to study its effect on a nearly as sophisticated brain structure as the human (Ma et al., 2005; Seu et al., 2009).

The rat and mouse models additionally provided evidence for involvement of genes in ADHD (Gainetdinov et al., 1999; Simchon et al., 2010; Wallis et al., 2012; Zhuang et al., 2001; Zimmermann et al., 2015). This was done by measuring face-valid hyperactivity, inattentiveness, or impulsivity, and also looking for physiological and histological abnormalities in reverse and forward genetic approaches. Reverse genetic approaches are employed for disease candidate genes identified in human studies; the orthologues of the observed human genes are manipulated in the animal model to evoke measurable phenotypes and to study their involvement on cellular and/or behavioural levels (Rivero et al., 2013; Wallis et al., 2012). Forward genetics use the opposite tactic by studying inbred animal strains for ADHD-associated conducts, and analysing their protein expression pattern and behaviour (Dimatelis et al., 2015; Li et al., 2007b; Womersley et al., 2015). In reverse genetics approaches, early studies proved the involvement of candidate genes from association studies in ADHD, e.g. by showing ADHD-like behavioural phenotypes in *Dat1* knock-out mice and the coloboma mouse showing a mutation in *Snap25* (Giros et al., 1996; Heyser et al., 1995). Most knock-out and transgenic mouse models targeted genes

involved in dopamine transmission. These mutant models provide an excellent opportunity to evaluate the contribution of dopamine-related processes to brain pathophysiology, to analyse the neuronal circuits and molecular mechanisms involved in the action of ADHD medication, and to test novel treatments for ADHD; for review see (Leo and Gainetdinov, 2013). More recent studies of a mouse model containing a null mutation of the latrophilin 3 gene (*Lphn3*), which showed a hyperactive phenotype in an open field test, revealed that this ADHD candidate gene is involved in gene expression regulation of monoamine signalling genes, such as dopamine and serotonin receptors and transporters, neurotransmitter metabolism genes, and neural development genes (Wallis et al., 2012). Additionally, actin depolymerising factor and n-cofillin double mutant mice displayed hyperlocomotion, impulsivity, impaired working memory, and disturbed morphology of striatal excitatory synapses, accompanied by strongly increased glutamate release (Zimmermann et al., 2015). Of note, the hyperlocomotion and impulsivity were reversed by methylphenidate (Zimmermann et al., 2015).

While reverse genetics approaches are most often applied in ADHD research to date, forward genetics has led to new models to study ADHD, like the spontaneous hypertensive rat (SHR), which was created to study hypertension by inbreeding albino Wistar rats showing elevated blood pressure (Okamoto and Aoki, 1963). These rats also showed deficits in attention, and ADHD-associated conducts, such as increased impulsivity and hyperactivity, and are therefore widely employed as a model for ADHD (Li et al., 2007b; Williams et al., 2009a, b, c). Studies of the molecular and cellular characteristics of this forward genetic model have provided evidence that proteins involved in energy metabolism, neurotransmitter function, neural development, and myelination are differently expressed in the striatum and PFC (Dimatelis et al., 2015; Womersley et al., 2015). The observed changes in the striatal energy metabolism support the neuroenergetics hypothesis of Killeen and colleagues, which states that inadequate neuronal energy supply can lead to ADHD symptoms (2013). Moreover, the GABAergic system was shown to be involved in ADHD-like behaviour of SHR rats by investigating the physiological response of GABA on hippocampal slices and finding GABA significantly altered during early-life stress in these animals (Sterley et al., 2013a, b).

Novel animal models for the study of ADHD include zebrafish (*Danio rerio*) and the fruit fly (*Drosophila melanogaster*) (Lange et al., 2012; van der Voet et al., 2016). These models have the great advantage of being relatively inexpensive, having a wide range of genomic tools available, and being highly suitable for fast, high throughput studies of (candidate) genes. The combination of those characteristics, with the availability of valid and quantifiable phenotypic readouts for ADHD-relevant traits makes these animal models a potent addition to non-invasive studies in humans. Even though both zebrafish and fruit fly diverged from the human lineage early in evolution, there is still a high level of conservation of neuronal genes; for example, in a screen for intellectual disability genes, more than 70% of candidate genes had an unambiguous orthologue in the fruit fly (Oortveld et al., 2013; van

der Voet et al., 2014). Also genes involved in the phenotypic manifestation of hyperactivity were crucial during early evolution and thus can be found in species with a much simpler nervous system. Several studies show the strength of such models. In zebrafish, e.g. the orthologues of the human ADHD candidate genes *LPHN3, PER1,* and *PER2* have been studied and shown to cause hyperactivity (Huang et al., 2015; Lange et al., 2012; Wang et al., 2015a). The *lphn3.1* mutant fish showed, besides the behavioural phenotypes, misplaced dopamineric neurons in the brain (Lange et al., 2012). Functional studies on *Per2* null mutants in zebrafish showed differential expression of the circadian clock genes *aanat2* and *bmal1b* and indicated the involvement of *Per2* in the circadian regulation (Wang et al., 2015a). In addition, *per1b* mutant fish display hyperactive and impulsivity-like behaviours and low levels of dopamine, of which hyperactivity could be rescued by ADHD medication (Huang et al., 2015). It could be shown that the circadian clock has direct influence on the structure and abundance of dopaminergic neurons and lower expression of transcription factors directly regulating development, maintenance and differentiation of these neurons (Huang et al., 2015). In the fruit fly, manipulation of the orthologues of the dopamine-related genes *DAT1* and *LPHN3*, caused characteristic darkness-dependent hyperactivity, an ADHD-like behaviour (van der Voet et al., 2016). Also in those models, ADHD medication was able to reverse the behavioural phenotype (van der Voet et al., 2016). Finally, the vacuolar protein sorting-associated protein 4A (*VPS4A*) in the striatal node was significantly associated with dysfunctional reward, a cognitive (e.g. poor inhibitory control or timing estimation), but also affective (e.g. delay aversion or hyposensitivity to reward) deficit observed in ADHD (Jia et al., 2016). The orthologue was studied in the fruit fly, showing that flies with an overexpression of *Vps4* have a reduced overall activity similar to a *Drd1* knockout fly, while a knockdown of *Vps4* leads to hyperactivity, suggesting the involvement of *VPS4A* in the regulation of *DRD1*-mediated activity (Jia et al., 2016).

At the level of animal models, the integration of knowledge from models of different evolutional complexity will optimally support the elucidation of gene to disease pathways, as it is already apparent from the initial study of LPHN3 orthologues across mouse, zebrafish, and fruit fly.

Modelling psychiatric disorders at the cellular level by using human induced pluripotent stem cell (hiPSC)-derived neurons

ADHD is a multifactorial disorder, and in most patients, several to multiple genetic factors are likely to contribute to disease. Animal models are mostly based on highly penetrant single gene mutations, which limits the translation of findings from molecular and cellular levels to the human situation. A human model, especially one derived from a patient him-/herself, might therefore be preferable in certain situations. Until recently, the only way to study cellular processes and molecular pathways in patient brain cells was through post-mortem material. Due to the scarceness of available post-mortem brain tissues, only a very limited

number of studies was performed to date, concentrated on demonstrating an influence of ADHD-related genetic variants on gene expression (Brookes et al., 2010; Brookes et al., 2007; Hawi et al., 2013b; Weber et al., 2015). Fortunately, cell reprogramming technology has been developed throughout the past decade, which provides a powerful tool to simulate neural developmental processes *in vitro* in a petri dish (Brennand et al., 2015b; Takahashi et al., 2007; Yu et al., 2007). Neurons from both healthy individuals and psychiatric patients can be derived by 1) reprogramming human skin fibroblasts or blood mononuclear cells into induced pluripotent stem cells (iPSCs) and then differentiating these into neurons (Zhang et al., 2013), or by 2) directly reprogramming skin fibroblasts into neural stem cells or neurons (Brennand and Gage, 2012; Pang et al., 2011; Vadodaria et al., 2015). The human induced pluripotent stem cell (hiPSC)-derived neurons from a patient then have a genetic background identical to the disorder state, and thereby provide the possibility to characterize the effects of the cocktail of genetic perturbations leading to disease in this patient (Brennand et al., 2015a; Lim et al., 2015; Madison et al., 2015; O'Shea and McInnis, 2015; Wang et al., 2015b). Such effects may be specific to cell types and/or specific developmental stages (Duan, 2015; Hockemeyer and Jaenisch, 2016). Moreover, a temporal analysis of disease initiation and progression can be performed in the cell type(s) most relevant to a disorder (Brennand et al., 2012). For example, studying hiPSC neurons from patients with disease-associated large CNVs provides the opportunity to perform comprehensive molecular analyses of the effects of these large CNVs in several cell lineages (Urban and Purmann, 2015). Until now, several studies were published using hiPSC-derived neurons from patients with ASD, schizophrenia, or bipolar disorder (Ananiev et al., 2011; Brennand et al., 2011; Chiang et al., 2011; Marchetto et al., 2010; Sheridan et al., 2011; Urbach et al., 2010; Wen et al., 2014). For example, hiPSC-derived neurons from patients with schizophrenia were shown to have synapse deficits and transcriptional dysregulation (Wen et al., 2014). Additionally, by comparing hiPSC-derived neurons from patients with bipolar disorder and controls, alterations in key components of the microRNA processing pathway were identified, potentially altering neuronal cell fate determination (O'Shea and McInnis, 2015). To overcome confounding effects of variable genetic backgrounds, when comparing cells from patients to those from healthy controls, genome-editing technology can be applied (Duan, 2015), such as Zinc-finger nucleases (ZFN) (Reinhardt et al., 2013), transcription activator-like effector nucleases (TALEN) (Wen et al., 2014), and recently developed clustered regularly interspaced short palindromic repeats (CRISPR) (Liu et al., 2016; Ran et al., 2013; Wang et al., 2015b). Such genome-editing enables the generation of isogenic hiPSC-derived neurons that differ only at the genetic site of interest (Bedell et al., 2012; Choi et al., 2013; Ding et al., 2013). In this way, specific mutations can be either introduced in control cells or corrected in patient cells to investigate causality (Duan, 2015; Hendriks et al., 2016).

Beyond mechanistic insights, hiPSC neurons might also serve as a platform for high throughput screening to identify novel therapeutics for psychiatric disorders (Schadt et al., 2014). For example, the ability to test drugs to rescue synaptic deficiency in Rett syndrome neurons has been demonstrated (Brennand et al., 2012). Generally, it is suggested that these cell models are suited for longitudinal observations, studying on-off-medication effects, and investigating the mechanisms of comorbid disorders in individual patients (O'Shea and McInnis, 2015).

A current limitation of hiPSC-based models is the high heterogeneity of the derived differentiated neuronal populations (Gore et al., 2011; Hu et al., 2010; Lister et al., 2011; Osafune et al., 2008). Although almost pure glutamatergic neurons can be differentiated from hiPSCs, the differentiation efficiency for other types of neurons, such as dopaminergic, GABAergic, serotonergic, or cholinergic neurons, is relatively low, which can cause variability in the outcome of *in vitro* experiments (Hu et al., 2016; Liu et al., 2013b; Swistowski et al., 2010; Vadodaria et al., 2015; Zhang et al., 2013). Therefore, ideally a comparison of several hiPSC-derived neuronal lines from multiple patients should be performed (Brennand and Gage, 2012). It seems advantageous to model the psychiatric disorders in a pure, specified type of neuron; however, it is also reasonable to carry out investigations at the network level in different types of neurons interacting, better mimicking the multidimensional integration in the brain through 3D culture systems or brain 'organoids', which are currently being developed (Kim et al., 2015; Lancaster et al., 2013).

Based on the above, it is clear that the reprogramming technology will revolutionize our use of model systems. Although still very much under development, and not yet applied to ADHD risk genes, first results for other psychiatric disorders already suggest that valuable findings about molecular and cellular pathways from gene to disease can be derived from iPSC-derived neurons.

Conclusion

Results from studies described in this review show that imaging genetics approaches are highly suitable to provide more insight into the pathways from gene to behaviour via the brain in ADHD. However, this field is clearly still in its early stages. Inconsistency of findings, due to the use of relatively small sample sizes, clinical and biological heterogeneity of ADHD, methodological differences in study design, and analysis methodology, make it difficult, yet, to draw firm conclusions about effects of genes on brain morphology, function, and connectivity. Individual genes need to be investigated more extensively and in larger samples, and additional genes need to be studied – preferably focussing on those implicated in ADHD through genome-wide, hypothesis-free approaches. We emphasize that a combination and integration of imaging genetics studies with complementary approaches at different levels of biological complexity - including bioinformatics as well as cell and animal models - will be necessary to fully map the biological pathways from gene to disease.

Acknowledgements

Funding: This works was supported by the Netherlands Organization for Scientific Research (NWO), i.e. the NWO Brain & Cognition Excellence Program (grant 433-09-229) and the Vici Innovation Program (grant 016-130-669 to BF). Additional support was received from the European Community's Seventh Framework Programme (FP7/2007 – 2013) under grant agreements n° 602805 (Aggressotype), n° 602450 (IMAGEMEND), and n° 278948 (TACTICS) as well as from the European Community's Horizon 2020 Programme (H2020/2014 – 2020) under grant agreements n° 643051 (MiND) and n° 667302 (CoCA). The work was also supported by grants for the ENIGMA Consortium (grant number U54 EB020403) from the BD2K Initiative of a cross-NIH partnership, and by the ECNP Network ADHD across the Lifespan.

Conflict of interest

None of the authors report conflicts of interest. Barbara Franke discloses having received educational speaking fees from Merz and Shire.

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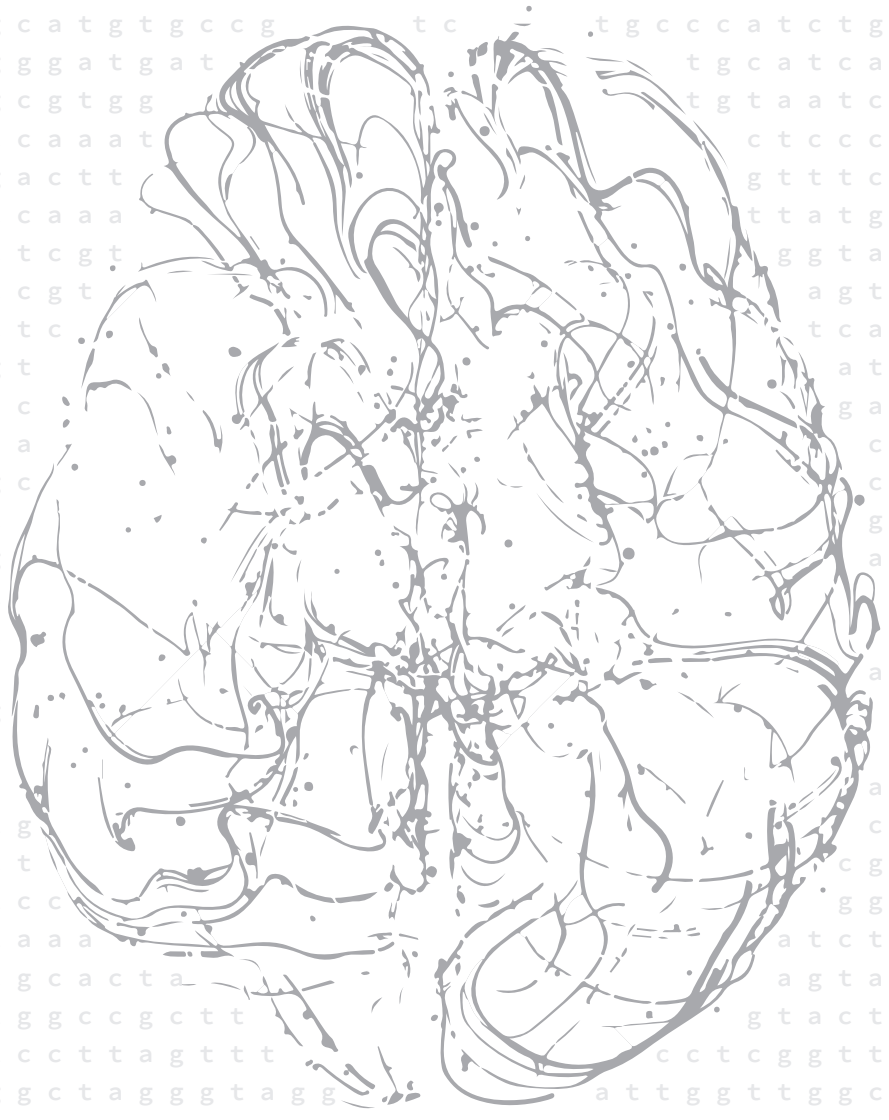

CHAPTER 9

Converging evidence does not support *GIT1* as an ADHD risk gene

Published as:
M. Klein*, M. van der Voet*, B. Harich, K. J. van Hulzen, A. M. Onnink, M. Hoogman, T. Guadalupe, M. Zwiers, J. M. Groothuismink, A. Verberkt, B. Nijhof, A. Castells-Nobau, S. V. Faraone, J. K. Buitelaar, A. Schenck, A. Arias-Vasquez, and B. Franke (2015). "Converging evidence does not support *GIT1* as an ADHD risk gene." Am J Med Genet B Neuropsychiatr Genet **168**(6): 492-507.
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Abstract

Attention-Deficit/Hyperactivity Disorder (ADHD) is a common neuropsychiatric disorder with a complex genetic background. The G protein-coupled receptor kinase interacting ArfGAP 1 (*GIT1*) gene was previously associated with ADHD. We aimed at replicating the association of *GIT1* with ADHD and investigated its role in cognitive and brain phenotypes. Gene-wide and single variant association analyses for *GIT1* were performed for three cohorts: (1) the ADHD meta-analysis data set of the Psychiatric Genomics Consortium (PGC, N=19,210), (2) the Dutch cohort of the International Multicentre persistent ADHD CollaboraTion (IMpACT-NL, N=225), and (3) the Brain Imaging Genetics cohort (BIG, N=1,300). Furthermore, functionality of the rs550818 variant as an expression quantitative trait locus (eQTL) for *GIT1* was assessed in human blood samples. By using *Drosophila melanogaster* as a biological model system, we manipulated Git expression according to the outcome of the expression result and studied the effect of *Git* knockdown on neuronal morphology and locomotor activity. Association of rs550818 with ADHD was not confirmed, nor did a combination of variants in *GIT1* show association with ADHD or any related measures in either of the investigated cohorts. However, the rs550818 risk-genotype did reduce *GIT1* expression level. *Git* knockdown in *Drosophila* caused abnormal synapse and dendrite morphology, but did not affect locomotor activity. In summary, we could not confirm *GIT1* as an ADHD candidate gene, while rs550818 was found to be an eQTL for *GIT1*. Despite GIT1's regulation of neuronal morphology, alterations in gene expression do not appear to have ADHD-related behavioral consequences.

Keywords: *GIT1*, ADHD, brain imaging genetics, eQTL, *Drosophila melanogaster*

Introduction

Attention Deficit/Hyperactivity Disorder (ADHD) is a common and highly heritable neuropsychiatric disorder (heritability 70-80% (Burt, 2009; Faraone et al., 2005)), with prevalence rates of 5-6% in childhood (American Psychiatric Association, 2013; Polanczyk et al., 2007). Clinically, ADHD is characterized by two core symptom domains: inattention and hyperactivity/impulsivity (American Psychiatric Association, 2013). At least 15% and up to 60% of all patients diagnosed in childhood still meet full ADHD criteria when they reach adulthood; prevalence rates of persistent ADHD in adults range between 2.5 and 4.9% (Simon et al., 2009). The clinical manifestation of adult ADHD may differ from that of childhood ADHD, i.e. by less obvious symptoms of hyperactivity and impulsivity (Buitelaar, 2011; Haavik et al., 2010). However, adult individuals with ADHD might be the most severe cases, given the lifelong impairment (Dalsgaard et al., 2015; Franke et al., 2012).

Despite its high heritability, identifying ADHD risk genes has been difficult (Franke et al., 2009a; Gizer et al., 2009), Due to the disorder's complex genetic background (Franke et al., 2012). Because of the high prevalence of ADHD in the population, the search for genetic factors has mainly focused on common genetic variants (mainly single nucleotide polymorphisms (SNPs)) that occur quite frequently and have generally small effect sizes (Li et al., 2014; Neale et al., 2010b). Hypothesis-free genome-wide association studies (GWAS) have thus been the main approach to study the genetics of ADHD during the last ten years. However, with eleven GWAS published to date (Hinney et al., 2011; Lasky-Su et al., 2008a; Lasky-Su et al., 2008b; Lesch et al., 2008; Mick et al., 2010; Neale et al., 2008; Neale et al., 2010a; Sanchez-Mora et al., 2014; Sonuga-Barke et al., 2008; Stergiakouli et al., 2012; Yang et al., 2013), no genome-wide significant hit has yet been identified for ADHD (Li et al., 2014; Neale et al., 2010b). A review of the first five hypothesis-free GWAS approaches for ADHD reported only limited overlap between the different studies, except for an association with cadherin 13 (*CDH13*) (Franke et al., 2009b). So far, only a handful of susceptibility genes have been identified through meta-analysis, all of which confer only small increases in disease risk (Gizer et al., 2009). Recently, the G protein-coupled receptor kinase interacting ArfGAP 1 gene (*GIT1*; Gene ID 28964), was suggested as a novel candidate gene for ADHD (Won et al., 2011). The *GIT1* gene comprises 21 exons and spans 16,123 base pairs. It is located on chromosome 17p11.2 and plays an important role in the regulation of cell migration (Penela et al., 2014), neurite outgrowth (Albertinazzi et al., 2003; Za et al., 2006) and synapse formation (Kim et al., 2003; Menon et al., 2010; Saneyoshi et al., 2008; Segura et al., 2007; Zhang et al., 2003; Zhang et al., 2005). In this, the finding of association of *GIT1* with ADHD fits well with earlier work of our group, showing convergence of top-findings from five genome-wide association studies in ADHD on the biological process of neurite outgrowth (Poelmans et al., 2011). Out of 27-tested single nucleotide polymorphisms (SNPs), the intronic SNP rs550818 in the *GIT1* gene was associated with ADHD in a Korean childhood sample (N=388; adjusted odds ratio

= 2.66) (Won et al., 2011). The authors reported that the minor allele of this SNP caused a reduction of GIT1 transcription in a luciferase reporter assay in HEK293 cells, indicating that it is a functional variant (Won et al., 2011). In the same report, *Git1*-deficient mice displayed ADHD-like symptoms, such as hyperactivity, but also enhanced theta rhythms, and impaired memory. All of these symptoms were reversed by amphetamine, a stimulant medication used for ADHD treatment (Won et al., 2011). However, the association between the SNP rs550818 and ADHD risk was not replicated in a Brazilian childhood and adolescent ADHD sample (N=646) (Salatino-Oliveira et al., 2012). To our knowledge, no other replications of the finding has been published yet, although a recent review listed the GIT1 association as a reproducible genetic association for ADHD (Hawi et al., 2015).

In the current study we investigated the role of *GIT1* (including SNP rs550818) in ADHD risk and related traits. First, we attempted to replicate the association between *GIT1* and ADHD in the largest data set available, the Psychiatric Genomics Consortium's (PGC; http://www.med.unc.edu/pgc/) ADHD data (N=19,210). We then assessed the effect of *GIT1* variation on ADHD-related neurocognition, brain volume measures and white matter integrity in adult ADHD patients and controls. We further examined whether SNP rs550818 alters *GIT1* mRNA expression in blood cells from patients with ADHD and controls. Lastly, we characterized the effects of downregulating expression of *Git* in *Drosophila melanogaster*, using synaptic and dendritic morphology and locomotor activity as read-outs.

Material and Methods

Cohorts

PGC ADHD meta-analysis

Data from nine studies including 5,621 cases and 13,589 controls were available for analysis. Samples were of Caucasian or Han Chinese origin and met diagnostic criteria according to the DSM-IV (**Supplementary Table 1**). The meta-analytic data used in this study were available as summary statistics, including genome-wide SNP data with corresponding p-values and odds ratios.

Dutch cohort of the International Multicentre persistent ADHD CollaboraTion (IMpACT-NL)

A total of 225 individuals (115 adult ADHD patients, 110 healthy control subjects matched for age, gender, and IQ) from IMpACT-NL (Franke and Reif, 2013; Franke et al., 2010a) participated in this study. Participants were recruited from the department of Psychiatry of the Radboud university medical center in Nijmegen or through advertisements. Patients were included if they met DSM-IV-TR criteria for ADHD in childhood as well as in adulthood. Participants had a mean age of 37.42 years (range 18-63), and 43.1% of the sample was male. For genetic data analysis, subjects were not allowed to be genetically related to each other. The study

was approved by the regional ethics committee. Written informed consent was obtained from all participants. A more detailed description of the IMpACT-NL cohort can be found in the supplementary material.

Brain Imaging Genetics Study (BIG)

The study sample consisted of healthy adult volunteers taking part in the diverse studies conducted at the Donders Institute for Brain, Cognition and Behaviour in Nijmegen, The Netherlands (Franke et al., 2010b). Genome-wide genotyping data and structural Magnetic Resonance Imaging (MRI) data was available for 1,300 subjects (Guadalupe et al., 2014; Hoogman et al., 2014). Participants were highly educated (80% with a bachelor student level or higher), of Caucasian descent, and had no self-reported neurological or psychiatric history. The mean age was 22.9 years (range 18-40 years), and 42.7% of the participants were males. All participants gave written informed consent and the study was approved by the regional ethics committee.

Demographic characteristics of the PGC, IMpACT-NL, and BIG cohorts are presented in **Table 1**.

Table 1. Demographic characteristics of the different cohorts.

	PGC (N = 19,210)	IMpACT-NL (N = 225)	BIG (N = 1,300)
Age [a]	NA	37.42 (10.94), 18-63	22.9 (3.82), 18-40
Gender	NA	43.1% male	42.7% male
Cases/controls	5,621/13,589	115/110	---

[a] Data are shown as mean (standard deviation), minimum – maximum.

Neuropsychological data

Data on cognitive functioning was available for participants of the IMpACT-NL cohort. They were assessed with a neuropsychological test battery composed to cover multiple cognitive domains earlier found affected in ADHD (Mostert et al., submitted). This included executive functioning, timing of motor output, reaction time, delay aversion, impulsivity, inhibition, attention, vigilance, working memory, motor speed, and set shifting. The neuropsychological tests were always administered in the same order across ADHD patients and healthy controls. The following tasks and variables were selected for association analyses with the *GIT1* locus, because related tasks were either studied by Won and colleagues (continuous performance task; (Won et al., 2011)), or were affected in *Git1* knockout mice (working memory (Won et al., 2011)): (1) sustained attention dots (SAD) task ((Huijbregts et al., 2008), variables: mean series completion time, standard deviation (SD) series completion time, SD series errors and the response bias) and (2) Digit span task ((Wechsler, 1997), variables: raw scores on forward and backward condition). Additionally, we explored the effect of the *GIT1* locus on the

following tasks and variables, because performance on these cognitive domains was shown to be different between ADHD patients and controls in our IMpACT-NL cohort (Mostert et al., submitted): (1) Flanker task ((Huijbregts et al., 2002), variable: total SD of reaction time (RT)), (2) Sustained Attention to Response Task (SART, (Smit et al., 2004), variable: SD of RT for hits), (3) Delay discounting task ((Dom et al., 2006), K100), and (4) Trail-making task ((Kortte et al., 2002), variables: time to complete part A and B). The following variables were log-transformed to achieve a normal distribution: SAD task standard deviation (SD) series completion time, SAD task SD series errors, SART SD of reaction time (RT), delay discounting task K100. For more detailed information on the tasks and variables see **Supplementary Table 2**.

Neuroimaging, MRI acquisition and data processing

Because altered brain volumes have been consistently found to be associated with ADHD (Castellanos et al., 2002; Frodl and Skokauskas, 2012), and *Git1* was shown to affect neurite outgrowth (Albertinazzi et al., 2003; Za et al., 2006), spine morphogenesis (Segura et al., 2007; Zhang et al., 2005), and synapse formation (Kim et al., 2003; Menon et al., 2010; Saneyoshi et al., 2008; Segura et al., 2007; Zhang et al., 2003; Zhang et al., 2005) in mice, we aimed to investigate the role of genetic variation within the *GIT1* locus on total brain volume, gray and white matter volume and white matter integrity in two different cohorts.

IMpACT-NL

T1-weighted MRI images were acquired previously and details of acquisition and processing are described in the supplementary material and elsewhere (Onnink et al., 2014). For 203 samples (101 ADHD patients and 102 healthy controls) both MRI and genetic data was available.

BIG

Anatomical T1-weighted whole brain MPRAGE scans were either acquired at a 1.5 Tesla scanner (Sonata and Avanto, Siemens Medical Systems, Erlangen, Germany) or at a 3 Tesla scanner (Trio and TrioTim, Siemens Medical Systems, Erlangen, Germany) at the Donders Centre for Cognitive Neuroimaging (Nijmegen, The Netherlands). The imaging protocols of the T1 scans included slight variations, because images were acquired during several studies. Details of these variations on the protocol used in the IMpACT-NL study and parameters are described in the supplementary material and elsewhere (Hoogman et al., 2014). For 1,300 subjects both MRI and genetic data were available.

Genetic data

PGC

We obtained access to genome-wide summary statistics from the most recent PGC ADHD meta-analysis. Detailed procedures of DNA isolation, whole-genome genotyping and imputation were described previously (Neale et al., 2010b). Shortly, genome-wide data was obtained from different genotyping arrays (**Supplementary Table 1**) and was imputed using 1000 Genomes data as a reference panel (Phase I integrated variant set release (v3) in NCBI build 37 (hg19) coordinates) for autosomal SNPs (Genomes Project et al., 2010). Meta-analytic data were processed through a stringent quality control pipeline applied at the PGC (Neale et al., 2010b).

IMpACT-NL

From all IMpACT-NL participants, DNA was either isolated from saliva using Oragene containers (DNA Genotek, Ottawa, Ontario, Canada) or from EDTA blood samples according to manufacturer's protocol at the department of Human Genetics of the Radboud university medical center. Genome-wide genotyping of 235 IMpACT subjects (122 cases, 113 controls) was performed using the Human CytoSNP 12 version 2 genotyping BeadChip (Illumina Inc., San Diego, California, USA). Details on data quality control and imputation procedure can be found in the supplementary material.

BIG

DNA isolation, whole-genome genotyping, and imputation were described previously (Guadalupe et al., 2014; Hoogman et al., 2014). Shortly, saliva was collected using Oragene containers (DNA Genotek, Ottawa, ON, Canada). Whole genome genotyping was done using Affymetrix GeneChip SNP, 6.0 (Affymetrix Inc., Santa Clara, CA). For imputation, the 1000 Genomes data was used as a reference panel (Phase 1.v3 EUR (Genomes Project et al., 2010)) and the imputation of autosomal SNPs was done following the Enhancing Neuro Imaging Genetics Through Meta Analysis (ENIGMA) protocol (according to NCBI build 37 (hg19) coordinates; http://enigma.ini.usc.edu/).

Association of the *GIT1* locus with ADHD and ADHD-related quantitative traits

Association analyses between *GIT1*, ADHD, and related traits were done in two ways. First, we performed a single SNP association between the earlier described ADHD-risk SNP rs550818, ADHD status, and/or ADHD-related quantitative traits. Second, we analyzed the association of the *joint* effect of all common genetic variants in the *GIT1* locus with ADHD status and/or ADHD-related quantitative traits.

Single-SNP analyses

The SNP rs550818 lies within intron 20 of the *GIT1* gene on chromosome 17, at base pair position 27901975 (hg19/build 37). The A-allele has been reported to be the risk allele. The minor allele frequency (MAF) and the R^2 estimates for rs550818 in the different samples are shown in **Supplementary Table 4**.

For the PGC data, the association p-value for rs550818 and ADHD status was extracted from the summary statistics. For the IMpACT-NL sample, association analyses for the self-reported symptom counts (hyperactivity/impulsivity, inattentive and combined symptoms) and the *GIT1* locus were performed in cases only (N=115), given the known case-control differences for these phenotypes. We applied a linear regression with an additive genetic model and a missing data likelihood score test in SNPTEST (version 2.4.1) (Marchini et al., 2007). Age and gender were used as covariates for all analyses. For the neuropsychological data, analyses were performed in the same way, including age, gender, and diagnostic status as covariates in the model (N≥178). For the analysis of MRI-derived traits, age, gender, and total white matter volume (when analyzing gray matter) or total gray matter volume (when analyzing white matter) were included as covariates for the association analyses (N=203). Diagnostic status was not used as a covariate, because we found no differences in brain volume between ADHD patients and healthy controls (**Supplementary Table 5**). For the BIG sample (N=1,300), association analyses for the *GIT1* locus were performed using linear regression for total brain volume, gray and white matter by using genotypic data and the "linear" command in PLINK (version 1.07) (Purcell et al., 2007). Age, gender, magnetic field strength, and total white matter volume (when analyzing gray matter) or total gray matter volume (when analyzing white matter) were used as covariates. Association p-values for rs550818 were extracted from regression results of the individual analyses.

To test the effect of rs550818 genotype on local gray and white matter volumetric and integrity differences, we performed a voxel-based morphometry (VBM; (Ashburner and Friston, 2000)) analysis on the T1 (N=1,261) and DTI data (N=255) in the BIG cohort. The genotypes of SNP rs550818 were coded to represent a linear allelic additive effect (0, 1 or 2). Age, gender, and magnetic field strength were used as covariates. Gray and white matter cluster extent was analyzed separately and tested across the entire brain using a $P_{FWE} < 0.05$ and a cluster-forming threshold of $P_{uncorrected} < 0.001$ (Hoogman et al., 2014). Fractional anisotropy (FA) and mean diffusivity (MD) were tested in the same manner, except that FA comparisons were restricted to voxels having anisotropy > 0.1.

Gene-based analysis

The *GIT1* locus was defined as the *GIT1* gene ± 25 kb flanking regions in order to capture regulatory elements (Bralten et al., 2011). The gene range was selected according to the UCSC Genome Bioinformatics Site (http://genome.ucsc.edu/). Gene-based tests of the *GIT1* locus were performed using the offline version of the versatile gene-based test for genome-wide association studies (VEGAS) software (Liu et al., 2010). This program uses SNP names (rs-numbers) and p-values as input to estimate gene-based effects. The approach takes LD between markers in a gene into account by using simulations based on the LD structure of a custom set of reference individuals (Liu et al., 2010). As a reference panel we used genotypic data from BIG (Guadalupe et al., 2014) imputed with 1000 Genomes Phase 1.v3 EUR reference panel (Genomes Project et al., 2010). VEGAS assigns SNPs to autosomal genes according to their position in hg19/build 37. A corresponding gene list was downloaded from http://www.biomart.org/biomart/martview. Multiple testing was based on the number of simulations per gene and was set to 10,000.

For the PGC ADHD meta-analysis data set, SNPs were included in this analysis if they showed an imputation score (R^2) ≥ 0.6 and MAF ≥ 0.01 in unaffected subjects and Hardy-Weinberg equilibrium (HWE) $P > 10^{-6}$. Out of 126 common genetic variants within the *GIT1* locus, 97 SNPs had valid rs-numbers and were considered in the subsequent analysis (**Supplementary Table 3**). In the data from IMpACT-NL, we analyzed the association of the *GIT1* locus (52 SNPs) with self-reported symptoms counts (total number of symptoms, number of inattentive symptoms, number of hyperactive/impulsive symptoms), neuropsychological variables, and MRI derived traits, such as total brain volume and gray and white matter volume (**Supplementary Table 3**). Subsequent gene-based tests used the results from the individual regression analyses as input for VEGAS described above. For the data from the BIG cohort, we analyzed the association of the *GIT1* locus with MRI-derived traits, i.e. total brain volume, gray and white matter volumes. SNP data selected required an imputation score (R^2) ≥ 0.3 and MAF ≥ 0.01. Forty-three SNPs within the *GIT1* locus were considered in subsequent analyses (**Supplementary Table 3**). Gene-based tests of the *GIT1* locus were performed with the offline version of VEGAS using the results from the individual regression analyses as described above. The multiple testing-corrected p-value for significance of the analyses described above, derived from 10,000 permutations, was determined as 0.05 divided by the number of tested variables.

Power calculation

The Genetic Power Calculator (GPC) (Purcell et al., 2003) was used to define the power our samples had at either a range of genotype relative risks (GRR, for the PGC ADHD meta-analytic data, testing for case-control discrete trait) or additive QTL variances (for the IMpACT-NL and BIG cohort, testing for quantitative association) at α=0.05. We used a disease prevalence of 5% (as estimated by Polanczyk *et al*.(Polanczyk et al., 2007), and a multiplicative model (power calculation based on the allelic test). The actual risk allele frequencies of SNP rs550818 for the individual cohorts were included in the power analysis.

Functional characterization of *GIT1*: effect of rs550818 on *GIT1* mRNA expression

We specifically tested for the effect of rs550818 genotype on mRNA expression of *GIT1* in human blood samples from the IMpACT-NL cohort. From 148 consecutive IMpACT-NL participants blood samples for RNA isolation were collected in PAXgene Blood RNA Tubes (produced by QIAGEN GmbH for PreAnalytiX GmbH, Hombrechtikon, Switzerland) at the Radboud university medical center.

Validation of rs550818 genotype by TaqMan genotyping assay

Rs550818 genotypes from the genome-wide genotyping array were validated for the IMpACT-NL samples prior to this analysis. Allelic discrimination of rs550818 was performed using Taqman® SNP Genotyping assay (Life Technologies, Nieuwerkerk a/d IJssel, The Netherlands; Assay ID: C_2416538_10). For a detailed description of the TaqMan genotyping assay conditions see the supplementary material.

RNA isolation and cDNA synthesis

Total RNA was extracted from PAXgene blood RNA tubes at the department of Human Genetics of the Radboud university medical center using the Qiagen PAXgene Blood RNA Kit (produced by QIAGEN GmbH for PreAnalytiX GmbH) according to manufacturer's protocol. RNA integrity was assessed by gel electrophoresis. The cDNA was synthesized from 500 ng RNA in a reaction volume of 20 µl using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories B.V. Veenendaal, The Netherlands) according to manufacturer's protocol. For the expression analysis a 1/3.75 dilution was used.

Gene expression analysis using Taqman assays

GIT1 mRNA gene expression was assessed using Taqman gene expression analysis (Taqman assay Hs01063104_m1 for *GIT1* [Life Technologies]) according to manufacturer's protocol. Glucuronidase beta (*GUSB*), was taken along as reference gene (Taqman assay Hs00939627_m1 for *GUSB* [Life Technologies]). For a detailed description of the gene expression analysis conditions see the supplementary material. All measurements were performed in triplicate, and blanks were taken along as quality control during mRNA expression assessment. Results were analyzed with the 7500 Software v2.0.6 (Life Technologies) using an automatic threshold. Only samples with standard deviations of the triplicates ≤ 0.25 were considered for subsequent analysis, which resulted in 121 samples. As a calibrator sample the mean ΔCT of all control samples with the major genotype was used. Data was visualized using GraphPad prism (version 5.03), and the mean and a 95% confidence interval are shown.

Statistical analysis

GIT1 mRNA expression data was normally distributed (**Supplementary Figure 1**). We determined the effect of rs550818 genotype on *GIT1* mRNA expression based on three genotype groups (independent variable) using linear regression analysis with an additive genetic model. We also assessed whether *GIT1* mRNA expression levels differed between healthy controls and participants with ADHD using a two-tailed Student's t-test. All data analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM Corp. Released 2011, IBM SPSS Statistics for Windows, Version 20.0 Armonk, NY: IBM Corp.).

Functional characterization of *Git* in *Drosophila*

Genetics and breeding

Conditional knockdown of the *Drosophila GIT1* ortholog *Git* (CG16728) in all or specifically in multidendrite neurons was achieved with the UAS-GAL4 system (Brand and Perrimon, 1993) using promoter lines w; *UAS*-Dcr-2; *elav*-GAL4 and *477*-GAL4, *UAS*-mCD8::GFP; *ppk*-GAL4, respectively (Dietzl et al., 2007). The *Git UAS*-RNAi line (vdrc108123 *UAS-RNAi*^{Git}*/CyO*) and its genetic background control (vdrc60100) were obtained from the Vienna *Drosophila* RNAi Centre (VDRC, (Dietzl et al., 2007). For synapse and dendrite experiments, stock vdrc108123 was rebalanced with CyO-GFP to allow for selection of knockdown larvae. Crosses were cultured according to standard procedures at 28°C.

Neuronal morphology of synapses at the neuromuscular junction

Synapses at the type 1b neuromuscular junctions (NMJs) of muscle 4 were analyzed as described previously (Schuurs-Hoeijmakers et al., 2012). Male L3-stage larvae of the genotypes *UAS-RNAi*^{Git}*/UAS*-Dcr-2; +/*elav*-GAL4 and the respective control +/*UAS*-Dcr-2; +/*elav*-GAL4 were dissected following a dorsal midline incision (Brent et al., 2009). Dissected larvae were fixed in 3.7% paraformaldehyde for 25 min, washed in PBS containing 0.3% Triton X-100 (PBST), stained with 1:125 anti-brp (nc82), washed in PBST, and stained with 1:500 Goat anti-Mouse Alexa Fluor 488 and 1:25 anti-dlg1 antibody covalently coupled to Goat anti-mouse Alexa Fluor 568 IgG1 (Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA; Zenon® Antibody Labeling Kit, Life Technologies). The larvae were mounted in Prolong anti-fade Gold (Life technologies). Images were taken with a Zeiss Axio Imager Z2 microscope (63x magnification), subsequently stacked and synaptic area, branches and active zones were analyzed in Fiji (Schindelin et al., 2012; Schuurs-Hoeijmakers et al., 2012).. For the *Git* RNAi genotype at least 19 synapses and for the control genotype at least 29 synapses were analyzed. Statistical analysis was performed in Graphpad prism (version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

Dendritic morphology of class IV dendritic arborization neurons

Dissection and immunostaining was performed as described above, but for imaging the dorsal dendritic arborization C (ddaC) class IV dendritic arborization neurons larval were opened along the ventral midline (Brent et al., 2009). Genotypes analyzed were *Git* RNAi: *UAS-RNAi*^{Git}*/477*-GAL4, *UAS*-mCD8::GFP; +/*ppk*-GAL4), and the control: +/*477*-GAL4, *UAS*-mCD8::GFP; +/*ppk*-GAL4. The *477* and *ppk* promoters simultaneously drive RNAi and expression of mCD8::GFP in a tissue-specific manner. Antibodies used were 1:100 Rat anti-mCD8 primary antibody and 1:200 Goat-anti-Rat Alexa Fluor 488. Z-stack images were taken at a Zeiss LSM 510 confocal microscope with a 20x objective. Z-stacks were imported into NeuronStudio (version 0.9.92, http://research.mssm.edu/cnic/tools-ns.html) for generation of neuronal reconstructions and Sholl analysis (10 µm interval) (Wearne et al., 2005). Tracing files were analyzed with L-Measure (version v5.2, (Scorcioni et al., 2008)) and significance was analyzed using the Student's (equal variance) or Welch's t-test (unequal variance).

Drosophila locomotor activity

Locomotor activity of individual male flies was recorded with the *Drosophila* Activity Monitor (DAM) system (Trikinetics, Waltham, MA) (Catterson et al., 2010; Suh and Jackson, 2007) to assess whether *Git* pan-neuronal knockdown flies displayed hyperactive behavior or sleep regulation defects. Activity of 3–5 days old male flies was recorded over 4 days on a 12-h light:dark cycle and the average daily activity of at least 25 flies for each genotype was calculated. Locomotor activity data were analyzed in pySolo (Gilestro and Cirelli, 2009), modified to analyze activity and sleep (the latter defined as 5-min of inactivity (Rosato and Kyriacou, 2006)) between 120–540 min relative day and 840–1260 min relative night to reflect the stable locomotor activity in those intervals. Statistical analysis was performed in Graphpad prism (version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). T-tests were performed on summarized statistics.

Results

Association between the *GIT1* locus and ADHD

Demographic characteristics of the different cohorts are presented in **Table 1**. Testing whether variation in the *GIT1* locus (including SNP rs550818) altered ADHD risk we found that neither the SNP rs550818 (P=0.49; odds ratio 1.022; 95% confidence interval (CI) 0.959 - 1.088) nor the *GIT1* locus showed association with ADHD in the PGC ADHD meta-analysis data (N=19,210, P=0.465, **Figure 1**). Power analysis showed that the test was highly powered to detect an association with a genotype relative risk (GRR) ≥1.1 (for range of GRRs see **Supplementary Table 6**).

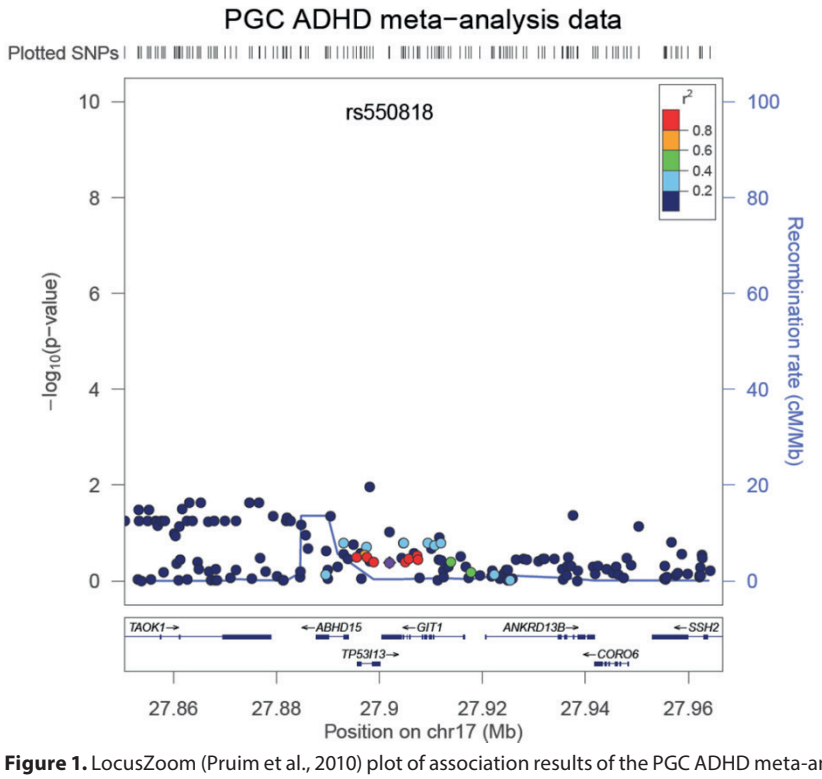

Figure 1. LocusZoom (Pruim et al., 2010) plot of association results of the PGC ADHD meta-analysis for *GIT1* including flanking regions of 25 kb on each site. Neither rs550818 (marked as purple index SNP), nor other SNPs within the gene range of *GIT1* showed association with ADHD susceptibility.

Association between the *GIT1* locus and quantitative measures related to ADHD in the IMpACT-NL cohort

We did not find an association of SNP rs550818 or the *GIT1* locus with self-reported hyperactivity/impulsivity or inattentive symptom counts (N=115, $P_{corrected} > 0.05$, and **Table 2**) in the IMpACT-NL cohort (for details on demographics see **Supplementary Table 7**). Previously, Won and others investigated the effect of rs550818 on sustained attention in a continuous performance task and they studied the effect of *Git1* deficiency in mice on working memory. Therefore, we tested the effect of rs550818 genotype and the *GIT1* locus on neuropsychological performance in the same cognitive domains. The association results between our genetic variables and outcomes of the SAD task (mean series completion time, SD series completion time, SD series errors, and the response bias) or the Digit Span task (forward and backward condition) were not significant (all P-values > 0.05, **Table 2**). Testing neuropsychological measurements in additional domains of cognitive functioning (reaction time, sustained attention, inhibition, impulsivity, delay aversion, motor control, and set shifting; for description of variables see **Supplementary Table 2**) did not provide

evidence for association with the rs550818 genotype or the *GIT1* locus ($P_{corrected\ for\ all\ tests} > 0.05$, **Supplementary Table 8**). However, power of these analyses was limited; the IMpACT-NL sample provided 32% power to detect an association explaining 1% of the variance (see more elaborate power analysis in **Supplementary Table 9**).

Table 2. Results of single-SNP and gene-based (rs550818 and *GIT1*) association analyses for self-reported ADHD symptom counts, Sustained Attention Dots (SAD) task and Digit Span task in the IMpACT-NL cohort.

Trait	Variable	N (HC/ADHD)	P rs550818	β	95% CI	P *GIT1* [c]
Self report symptom score [a]	Hyperactivity/impulsivity	-/115	0.413	0.134	-0.191 - 0.458	0.477
	Inattention	-/115	0.593	0.088	-0.237 - 0.413	0.944
	Total	-/115	0.395	0.140	-0.186 - 0.466	0.614
Sustained Attention Dots Task [b]	Mean series completion time	99/95	0.445	-0.091	-0.324 - 0.143	0.696
	Standard deviation series completion time*	99/95	0.142	-0.173	-0.405 - 0.059	0.563
	Standard deviation series errors*	99/95	0.439	-0.089	-0.316 - 0.138	0.808
	Response bias*	99/95	0.150	-0.163	-0.387 - 0.061	0.424
Digit Span Task [b]	Forward score raw*	100/98	0.511	-0.076	-0.304 - 0.152	0.433
	Backward score raw	100/98	0.941	0.008	-0.217 - 0.233	0.831

[a] Age and gender were used as covariates and 52 SNPs were considered for the gene-based analysis. [b] Age, gender and diagnostic status were used as covariates. 52 SNPs were considered in the gene-based analysis. [c] Effect sizes and 95% confidence intervals could not be estimated for the gene-based association tests.
* Variables that are significantly different between adult ADHD patients and healthy controls after correction for multiple testing (Mostert et al., submitted).

Association between the *GIT1* locus, brain volume and white matter integrity of microstructure

We investigated the effect of the *GIT1* locus on brain volume measurements in the case-control sample IMpACT-NL (N=203) and the population-based cohort BIG (N=1,300). Given the known involvement of *Git1* in neuronal development (Segura et al., 2007; Za et al., 2006; Zhang et al., 2005), we tested associations of genetic variation in *GIT1* with global brain measures for gray matter, white matter, and total brain volumes. None of these analyses yielded significant associations ($P_{corrected\ for\ all\ tests} > 0.05$, and **Table 3**). Additionally, we performed exploratory voxel-wise brain-wide analyses of gray and white matter volume, and of microstructural integrity in the BIG cohort for rs550818 to identify potential local effects of *GIT1* variation. Neither the VBM analyses for gray or white matter volume, nor the voxel-wise analyses for mean diffusivity and fractional anisotropy showed significant associations with rs550818 genotype (data not shown). While the IMpACT-NL sample again only provided limited power for this analysis (**Supplementary Table 9**), the analyses in the

BIG cohort were highly powered to detect associations explaining between 1% (>95%) and 0.5% (>72%) of variance (**Supplementary Table 9**).

Table 3. Results of single-SNP and gene-based (rs550818 and *GIT1*) association analyses for brain volumes in the IMpACT-NL and BIG cohort

	IMpACT-NL cohort [a]				BIG cohort [c]			
	P rs550818	β	95% CI	P *GIT1* [b,e]	P rs550818	β	95% CI	P *GIT1* [d,e]
Total brain volume	0.658	0.039	-0.134 - 0.211	0.563	0.897	0.511	-2.269 – 3.292	0.415
Total gray matter volume	0.622	-0.035	-0.175 - 0.105	0.497	0.970	0.069	-0.624 – 0.761	0.791
Total white matter volume	0.361	0.079	-0.084 - 0.229	0.154	0.934	0.150	-0.874 – 1.174	0.453

[a] N=203 (101 ADHD patients). Adult ADHD patients do not differ in brain volume from healthy controls (**Supplementary Table 5**). [b] 52 SNPs were considered for the gene-based analysis. [c] N=1,300. [d] 43 SNPs were considered for the gene-based analysis. Total brain volume is the sum of total gray and white volume. Age, gender, magnetic field strength, and gray matter when testing for white matter and vice versa were used as covariates. [e] Effect sizes and 95% confidence intervals could not be estimated for the gene-based association tests.

Functional characterization: effect of rs550818 on *GIT1* mRNA expression

Previously, it was reported that the minor allele (A) of the SNP rs550818 caused a reduction in luciferase transcription in HEK293 cells (Won et al., 2011). We therefore investigated this effect in blood samples of adult ADHD patients and healthy controls from the IMpACT-NL cohort. High quality RNA samples were available for 121 individuals (55 healthy controls and 66 individuals with ADHD); the G allele was the major allele in our European Caucasian sample. Indeed, SNP rs550818 genotype significantly affected *GIT1* mRNA expression in the total sample independent of diagnostic status (N=121, $b_{standardized}$=-0.220, P=0.015); carriers of the common allele (GG; N=63) had highest expression, while heterozygotes (GA; N=53) had intermediate expression and the carriers of the risk-associated genotype (AA; N=5) showed lowest expression (**Figure 2A**). *GIT1* mRNA expression levels did not differ significantly between healthy controls and participants with ADHD (t=1,559 df=119, P=0.1217) (**Figure 2B**).

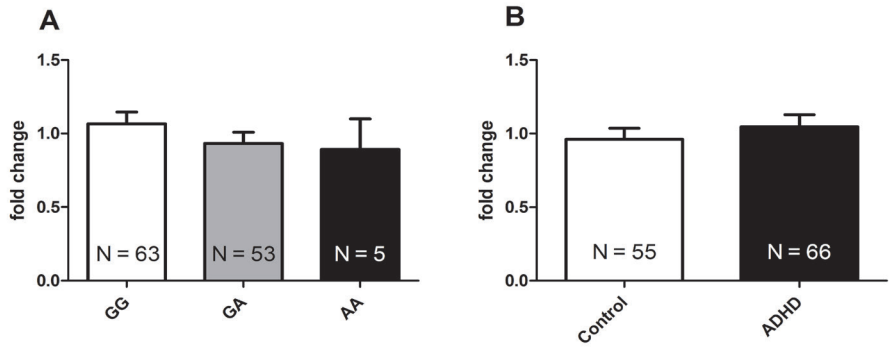

Figure 2. *GIT1* mRNA expression in human blood samples was dependent on rs550818 genotype but not diagnostic status in participants from the IMpACT-NL cohort. **A**) The minor A allele reduced *GIT1* mRNA expression in human blood samples (N=121, *b*=-0.116, $t_{(119)}$=-2.462, P=0.015, R^2=0.048). Bar charts represent mean and 95% confidence interval. **B**) *GIT1* mRNA expression fold change did not differ in healthy controls compared to individuals with ADHD (P=0.1217; two-tailed Student's t-test). Individuals with ADHD were distributed across the different genotype groups as following: N_{GG}=26, N_{GA}=26 and N_{AA}=3.

Functional characterization: effect of *Git* RNAi on neuronal morphology and locomotor activity in *Drosophila*

The fruit fly *Drosophila melanogaster* is a suitable model to study the behavioral and cellular consequences of genes associated to genetic disorders (van der Voet et al., 2014). To model the ADHD risk allele and validate the function of *GIT1* in neuronal morphology, we targeted the *Drosophila GIT1* ortholog, *Git*, using conditional RNA interference. The effect of the neuronal *Git* knockdown on synaptic organization was studied at the neuromuscular junction (NMJ). The *Drosophila* larval NMJ is a well-established synaptic model system that shares major features with central excitatory synapses in the mammalian brain (Koh et al., 2000) and has successfully been used for characterizing a number of *Drosophila* models of neurological diseases, including schizophrenia (Dickman and Davis, 2009) and intellectual disability disorders (Bayat et al., 2011; Liu et al., 2011; Schenck et al., 2003; Zweier et al., 2009). Pan-neuronal knockdown of *Git* resulted in a significant decrease in the number of neurotransmitter release sites, so-called active zones, per synaptic terminal compared to controls (0.87 fold, P=0.027), whereas the total area of the neuromuscular junction (NMJ) was not changed (P=0.96) (**Figure 3A+B**). Quantitative evaluation of synaptic terminal morphology revealed abnormal branching of synaptic terminals in the *Git* RNAi knockdown condition (**Figure 3B**). Both the number of branches and branching points were significantly increased at NMJs of the *Git* RNAi line when compared to control flies (1.49 and 1.86 fold, P=0.0002 and 0.0032, respectively).

Drosophila class IV dendritic arborization (da) neurons are complex and provide a good model for studying dendritic morphology (Jan and Jan, 2010). Knockdown of *Git* in these neurons induced abnormal dendritic complexity (**Figure 3C**). Quantification of the traced,

reconstructed neurons revealed a reduced number of branches, bifurcations, and terminal tips in the knockdown condition compared to control (0.63 fold, P=0.0003 for all three parameters) (**Figure 3D, Supplementary Figure 2**). The average branch length did not differ significantly (P=0.061), but the total branch path length was decreased in the mutant neurons (0.74 fold, P=0.0002) (**Supplementary Figure 2, Figure 3D**). These data suggest that *Git* knockdown results in a branching defect. Consistently, the maximum branch order was reduced (0.84 fold, P=0.017) and a Sholl analysis that plots the branch order as a function of soma distance, reveals a reduction in branch order throughout the neuron (**Figure 3D**). Other dendritic parameters, namely branch contraction and partition asymmetry, were not significantly different (**Supplementary Figure 2**).

We have recently demonstrated increased locomoter activity and decreased sleep in ADHD *Drosophila* models (van der Voet et al., 2015). We therefore assessed whether *Git* pan-neuronal knockdown flies also affect locomotor behavior. No defects in activity levels were found (day: P=0.4 and night: P=0.1, respectively; **Figure 3E**). Sleep of *Git* knockdown flies did also not differ from their genetic background controls (P=0.4 and P=0.2, respectively; **Figure 3E**). These data suggest that despite a role in regulating synapse and dendrite morphogenesis, *Git* knockdown does not cause increased locomotion.

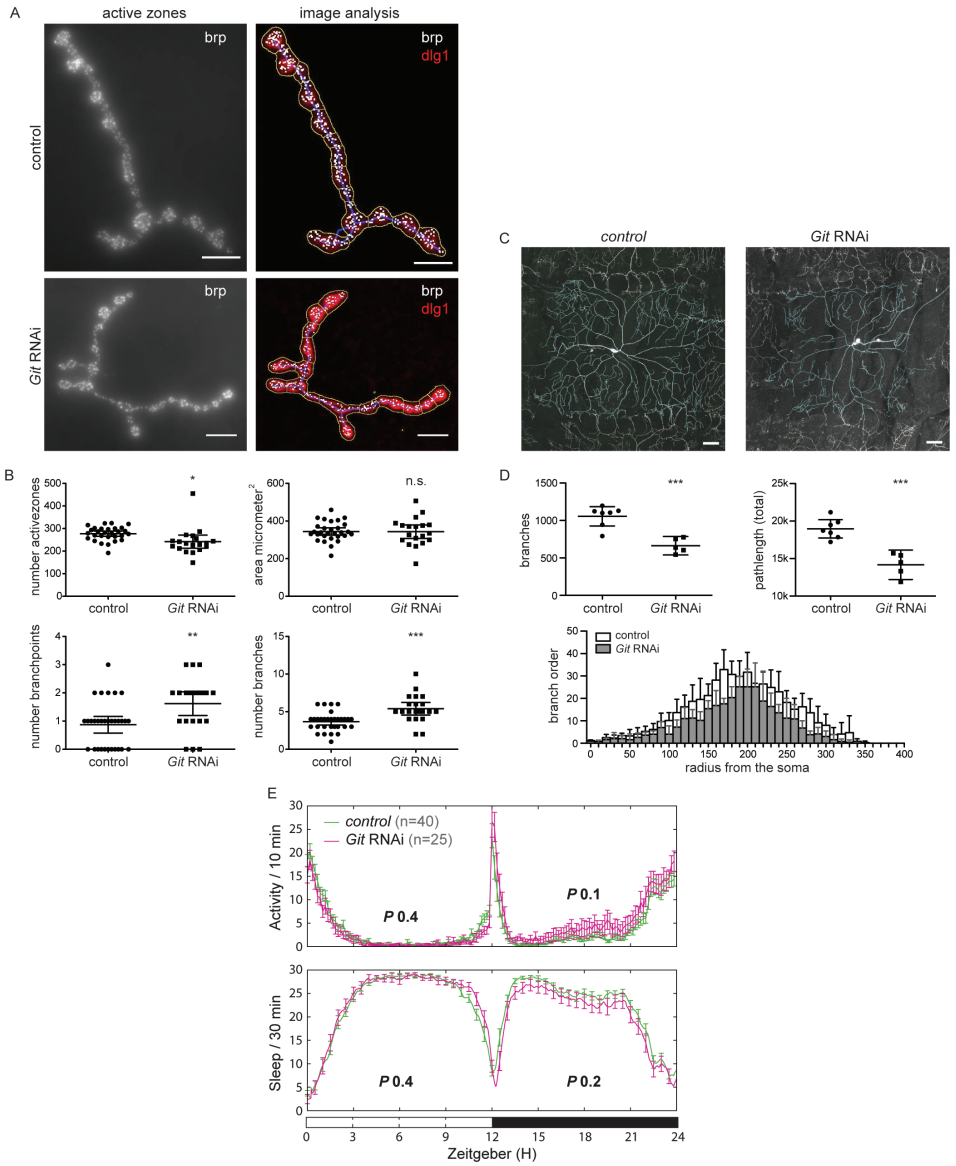

Figure 3. *Git* knockdown in *Drosophila* interfered with synapse and dendrite morphology, but did not alter locomotor activity. **A**) Representative *Drosophila* synaptic terminal at the neuromuscular junction (NMJ) for control and *Git* RNAi larvae. Overall morphology of synaptic terminals were visualized with an antibody against the disc large 1 (dlg1) protein, active zones, the presynaptic sites of neurotransmitter release, with an antibody against the active-zone component bruchpilot (brp). Each white foci represents one active zone. Images were quantitatively analyzed using an in house-developed Fiji macro (Schuurs-Hoeijmakers et al., 2012). Scale bar 10 µm. **B**) Quantitative analysis of NMJs showed a significant decrease in active zone count (P=0.027), increase of branch count (P=0.0002) and branching point count (P=0.0032), while the area was not different (P=0.96). Scatter plots represent individual measurements (*Git* RNAi N≥19 and control N≥29), mean and error bars indicate the 95% confidence interval. **C**) Representative *Drosophila* class IV da neurons show abnormal dendritic morphology in *Git* RNAi compared to wildtype control animals. Scale bar 50 µm. **D**) Quantitaive analysis of dendritic trees revealed that *Git* RNAi (N=5) reduces the number of branches (P=0.0003) and total branch path length (P=0.0002), compared to the control (N=7). Sholl analysis reveals that the branch order throughout the neuron is reduced. Scatter plots represent individual measurements. Error bars indicate the 95% confidence interval. **E**) Locomotor activity profiling of adult *Git* RNAi and control flies revealed normal activity or sleep parameters (values for day (Zeitgeber 0-12h, white bar) and night (Zeitgeber 12-24h, black bar) periods indicated). * P < 0.05; ** P < 0.01; ***P < 0.001. P-values were derived from two-sided Student's t-tests, except for not-normally distributed data, then a Wilcoxon-ranked test was performed.

Discussion

In the original publication of *GIT1* as a risk gene for ADHD, 27 SNPs in a 19 kb region encompassing the *GIT1* gene had been analyzed. Of those, eight SNPs had been shown to be polymorphic in a Korean childhood sample (N=388), and rs550818 was found associated with ADHD (Won et al., 2011). In addition, homozygous deficiency of *Git1* in mice resulted in increased locomotor activity (Won et al., 2011). In this study we performed a multilevel investigation of the role of the *GIT1* locus in ADHD risk and related traits (behavioral and MRI-derived) as well as functional characterization of the *GIT1* gene in humans and in *Drosophila*. Our results clearly show that the *GIT1* locus is not associated with ADHD risk, ADHD symptom counts, neuropsychological performance, or brain volume and white matter integrity variation in large human data sets. However, we demonstrated that rs550818 is indeed functional, as it lowered *GIT1* mRNA expression in human blood samples independently of ADHD diagnostic status. Using *Drosophila* as a model system, we showed that neuron-specific *Git* knockdown altered synaptic and dendritic morphology, whereas locomotor activity parameters remained unchanged.

Using the largest currently available ADHD sample, the PGC ADHD meta-analysis sample (N_{cases}=5,621, $N_{controls}$=13,589) we analyzed SNP rs550818 as well as the *combined* effects of all SNPs within the *GIT1* locus. Although our study had sufficient statistical power to detect an association, we were unable to replicate the initial finding by Won and coworkers. This is consistent with the results of an earlier replication attempt in a Brazilian childhood ADHD sample (Salatino-Oliveira et al., 2012). Despite the non-significant association, we showed that the effect is in the same direction as previously reported (Won et al., 2011), whereas the Brazilian study reported an odds ratio of 0.749, indicating an opposite directionality (Salatino-Oliveira et al., 2012). Importantly though, samples used in our and in the Brazilian study consisted (mainly) of participants of Caucasian ethnic origin, while all participants in the first study had an Asian ethnic background (Salatino-Oliveira et al., 2012; Won et al., 2011). Whereas allele frequencies of the present study and the Brazilian study (Salatino-Oliveira et al., 2012) are consistent with frequencies found in the European population (MAF=0.27 for allele A), frequencies in Asian populations – including the Korean one (Won et al., 2011) – strongly differ from this (MAF between 0.06 and 0.09). Therefore, the lack of replication can be difficult to interpret, as diverse genetic backgrounds and variable environmental

exposures may lead to distinct causal genetic variants in different populations (Campbell and Rudan, 2002).

Individuals with ADHD frequently display cognitive deficits, including impairments in inhibition, attentional processing, and increased reaction time variability (Castellanos et al., 2006; Kofler et al., 2013; Sonuga-Barke et al., 2010). A number of such cognitive domains has also been found impaired in *Git1*-deficient mice. For example, *Git1* knockout mice showed impaired spatial learning and memory in the Morris water maze task and impaired recognition memory during a novel-object recognition task (Won et al., 2011). Therefore, we tested the *GIT1* locus for association with cognitive performance in relevant domains. However, in concordance with the findings of Won and coworkers, who had applied a continuous performance test in the Korean childhood sample (Won et al., 2011), we did not find an effect of rs550818 or the entire *GIT1* locus on sustained attention in our adult ADHD sample, nor did neuropsychological performance in any of the other tested domains show association with *GIT1*. Additional cognitive deficits observed in *Git1* knockout mice, which were not tested in the current study, include impaired fear response and reduced adaptation to novel and changing environments (Menon et al., 2010; Schmalzigaug et al., 2009). Tasks quantifying fear response, like the eye blink component of the startle response (Davis, 2006; Hajcak et al., 2009), and those measuring reversal learning and tapping into adaptability might therefore be interesting phenotypes for future studies in humans in relation to genetic variation in the *GIT1* locus.

Git1 knockout mice exhibit alterations in dendritic length and spine density (Fiuza et al., 2013; Menon et al., 2010; Zhang et al., 2005). ADHD has been associated with volume differences in the brain (Onnink et al., 2014; van Ewijk et al., 2012), and we have shown that ADHD symptoms are associated with total brain volume in the general population (Hoogman et al., 2012). Thus, we investigated the role of *GIT1* in global and voxel-wise brain volume measures and microstructural integrity. However, we could not find an effect of *GIT1* on any brain measurements. In a way, this is consistent with the findings in mice, where changes of neuronal morphology did not translate into structural abnormalities observable at the macroscopic level in 3-month-old mouse brains in *Git1* knockout mice (Menon et al., 2010).

Won and colleagues had shown that the minor allele of rs550818 (A) reduced luciferase signal in an *in vitro* transcription assay (Won et al., 2011). *In vivo*, in human blood samples, we were able to confirm this effect of the A allele of rs550818, showing that *GIT1* mRNA expression was reduced in carriers of the minor allele. Generally, eQTLs can be specific to certain tissues, cells, anatomical regions and diseases (GTex Consortium 2013; (Emilsson et al., 2008). Therefore, our findings cannot necessarily be translated to other tissue types, e.g. the brain (McKenzie et al., 2014). However, a recent large study shows that there is also overlap between eQTLs from peripheral blood and eQTLs in brain (Wright et al., 2014), which implies

that some local regulatory variants might show ubiquitous effects (Kim et al., 2014). In the case of the *GIT1* eQTL, the fact that consistent effects have been found *in vitro* and *in vivo* might indeed indicate that effects are ubiquitous. However, this effect does not seem to be strong enough to modify brain structure, cognitive performance, or ADHD-related behavior.

Git1 is responsible for recruiting proteins to the synapse, and *Git1* knockout mice displayed decreased dendritic length and spine density (Menon et al., 2010; Zhang et al., 2005). A recent study identified *Drosophila Git* as a component of the active zone-associated cytomatrix and as a regulator of synaptic vesicle endocytosis and recycling (Podufall et al., 2014), although the actual number of active zones had not been evaluated in this study. Consistent with *Git* being a component of active zones, we did observe a mild but significant reduction in the number of active zones. We further showed that neuronal *Git* RNAi knockdown interferes with synaptic terminal branching and dendrite formation in *Drosophila*. This is consistent with earlier findings showing that various trafficking mutants of genes involved in organelle trafficking processes result in alterations of dendrite morphogenesis (Corty et al., 2009). Altogether, these observations support an important role for *Git* in synaptic and dendritic organization. Despite altered neuronal morphology, however, *Git* knockdown did not result in the locomotor hyperactivity that has been observed for other *Drosophila* models of ADHD-associated genes (van der Voet et al., 2015). Our knockdown is likely not to remove all of Git protein from the *Drosophila* brain. Also, the *GIT1* variant, even if homozygous, causes only a slight reduction in *GIT1* expression. Importantly, the two-fold higher spontaneous locomotor activity in an open-field test in *Git1* knockout mice (Won et al., 2011) was only observed in the homozygous condition with no detectable protein. Mice with a heterozygous deletion showed normal locomotor activity, in agreement with our findings in flies and humans.

At the cellular level, the effect of *GIT1* knockdown has been demonstrated in different model systems. We showed in human blood samples, that rs550818 affects *GIT1* gene expression. Interestingly, the cellular effects in the *Git1* knockout mouse model of Won and others seemed to be cell specific, as specifically inhibitory synaptic transmission was decreased (Won et al., 2011). Won and colleagues suggested that the resulting increase in neuronal excitability might contribute to the development of ADHD-like phenotypes. Although we demonstrated that genetic variation in the *GIT1* locus is not associated with ADHD in humans, we cannot rule out any other effects of the *GIT1* locus on different behavioral characteristics. The observed effect of *Git1* deficiency in mice on fear learning and adaptation to new environments, might be interesting starting points for future studies in humans.

The present findings should be viewed in light of several strengths and limitations. The main strengths of our study are its comprehensive approach on multiple levels and the use of the largest and well powered ADHD meta-analysis data set currently available. Moreover,

we did not only test association for a single SNP, but also investigated the *combined* effect of *all* SNPs within the *GIT1* locus available in our data sets. We also studied the role of the *GIT1* locus in various neuropsychological measures and investigated potential effects of *GIT1* on brain morphology in humans, in patients as well as a large population sample. Next to the association analyses, we also assessed the functional role of SNP rs550818 by mRNA expression analysis. For our functional analyses we used a novel and validated fly model for ADHD-related hyperactivity, which has been shown to be very useful in characterizing effects of ADHD candidate genes on synapse morphology and locomotor behavior (van der Voet et al., 2015). A clear weakness of our study was the limited size of our patient sample for the neuropsychological analyses, which might have been underpowered to reliably detect genetic effects in a relevant range of explained phenotypic variance. Additionally, the association of rs550818 with ADHD was originally identified in a childhood sample (Won et al., 2011), whereas our association analyses for neuropsychological and brain-related traits were performed in adult participants. This can be criticized as we know that differential genotype-phenotype association can exist at different ages and that genetic and neurocognitive mechanisms underlying ADHD may change throughout life (Greven et al., 2011; Larsson et al., 2011; Thissen et al., 2015). To overcome these limitations, it would be recommendable to also test for association with the number of ADHD symptoms in larger samples (of children) with ADHD. Furthermore, this study focused only on common genetic single nucleotide variants (SNVs), although it is known that these cannot completely explain the heritability of ADHD (Gratten et al., 2014). Therefore, rare genetic variation within the *GIT1* locus, be it single nucleotide or structural variants, might still play a role in ADHD. However, we already showed that an alteration of *GIT1* mRNA expression does – if not complete – not affect behavior. Even when *Git* is knocked down strongly in neurons, no behavioral changes in the model system were observed. Thus, we think it is unlikely that rare genetic variants within the *GIT1* locus will contribute to ADHD. Lastly, the gene-based testing methods we used did not provide us with effect size measures, which can help to better interpret the results of association findings.

In summary, our findings do not provide evidence for an impact of the *GIT1* locus on ADHD risk or the variation of ADHD-related traits in humans. Although rs550818 is associated with the variation of *GIT1* expression in blood, this does not appear to be a risk factor for ADHD. Therefore, *GIT1* is not supported as a candidate gene for this psychopathology, despite its reproduced and newly identified functional roles in neuronal morphology. Our study stresses the need for multi-level approaches in the study of genetic risk factors influencing the neurobiological mechanisms underlying ADHD etiology.

Acknowledgements

The authors wish to thank all participants who took part in the study. This work was carried out on the Dutch national e-infrastructure with the support of SURF Foundation. We thank Arjen Henkes for setting up the offline version of VEGAS. The summary statistics of the PGC ADHD meta-analysis used for the analysis described in this paper was obtained from the Psychiatric Genomics Consortium (PGC; https://pgc.unc.edu/).

This study is part of the international multicentre persistent ADHD collaboration (IMpACT). IMpACT unites major research centers working on the genetics of ADHD persistence across the lifespan and has participants in the Netherlands, Germany, Spain, Norway, the United Kingdom, the United States, Brazil and Sweden. Principal investigators of IMpACT are: Barbara Franke (chair), Andreas Reif, Stephen V. Faraone, Jan Haavik, Bru Cormand, Antoni Ramos Quiroga, Philip Asherson, Klaus-Peter Lesch, Jonna Kuntsi, Claiton Bau, Jan Buitelaar, Stefan Johansson, Henrik Larsson, Alysa Doyle, and Eugenio Grevet.

This work made use of the BIG (Brain Imaging Genetics) database, first established in Nijmegen, The Netherlands, in 2007. This resource is now part of Cognomics (www.cognomics.nl), a joint initiative by researchers of the Donders Centre for Cognitive Neuroimaging, the Human Genetics and Cognitive Neuroscience departments of the Radboud university medical centre and the Max Planck Institute for Psycholinguistics in Nijmegen. We wish to thank all persons who kindly participated in the BIG research.

Financial support

This study was supported by grants from the Netherlands Organization for Scientific Research (NWO), i.e. the NWO Brain & Cognition Excellence Program (grant 433-09-229) to A. Schenck and B. Franke, a Vici grant to B. Franke (grant 016-130-669), and by grants from the Netherlands Brain Foundation (grant 15F07[2]27) and BBMRI-NL (grant CP2010-33). M. van der Voet is supported by a Veni grant (grant 91614084). This research was also supported by the European Union's FP7 large scale integrated network Gencodys (HEALTH-241995), and by VIDI and TOP grants (917-96-346, 912-12-109) from NWO to A. Schenck.

The Psychiatric Genomics Consortium ADHD meta-analysis was supported by the following grants: US National of Institute of Health Grants R13MH059126, R01MH62873, U01MH085518 and R01MH081803 to S.V. Faraone, U01MH085515 to M. Daly K23MH066275-01 to J. Elia, and R01MH58277 to S. Smalley, Institutional Development Award to the Center for Applied Genomics from the Children's Hospital of Philadelphia to H. Hakonarson, Affymetrix Power Award, 2007 to B. Franke, NHMRC (Australia) and Sidney Sax Public Health Fellowship (443036) to S.E. Medland, Wellcome Trust, UK for sample collection to L Kent, UMC Utrecht Genvlag Grant and internal grants of Radboud University Medical Center to J.

Buitelaar, the Deutsche Forschungsgemeinschaft (KFO 125, SFB 581, GRK 1156 to K.P. Lesch, ME 1923/5-1, ME 1923/5-3, GRK 1389 to C. Freitag and J. Meyer, SCHA 542/10-3 to H. Schäfer) and the Bundesministerium für Bildung und Forschung (BMBF 01GV0605 to K.P. Lesch), National Natural Science Foundation of China (30800302 to Li Yang, 81071109 to Qiujin Qian), Chinese Ministry of Health grants (200802073 to Qiujin Qian), Ministry of Education Program for New Century Excellent Talents in University (NCET-11-0013 to Qiujin Qian), Ministry of Science and Technology grants (2007BAI17B03 to Bin Xie, Yufeng Wang).

The Cognomics Initiative is supported by the participating departments and centres and by external grants, i.e. the Biobanking and Biomolecular Resources Research Infrastructure (Netherlands) (BBMRI-NL), the Hersenstichting Nederland, and the Netherlands Organisation for Scientific Research (NWO). The research leading to these results also receives funding from the European Community's Seventh Framework Programme (FP7/2007– 2013) under grant agreements #602450 (IMAGEMEND) and #602736 (Aggressotype) and from the EU's Horizon 2020 Programme under grant agreement n° 643051 (MiND), from ERC- 2010-AdG 268800-NEUROSCHEMA, and from the National Institutes of Health (NIH) Consortium grant U54 EB020403, supported by a cross-NIH alliance that funds Big Data to Knowledge Centers of Excellence.

Conflict of interest

J. Buitelaar has been in the past 3 years a consultant to / member of advisory board of / and/or speaker for Janssen Cilag BV, Eli Lilly, Shire, Novartis, Roche and Servier. He is not an employee and not a stock shareholder of any of these companies. He has no other financial or material support, including expert testimony, patents, royalties.

In the past year, S. Faraone received income, travel expenses and/or research support from and/or has been on an Advisory Board for Pfizer, Ironshore, Shire, Akili Interactive Labs, CogCubed, Alcobra, VAYA Pharma, Neurovance, Impax, NeuroLifeSciences and research support from the National Institutes of Health (NIH). With his institution, he has US patent US20130217707 A1 for the use of sodium-hydrogen exchange inhibitors in the treatment of ADHD. In previous years, he received consulting fees or was on Advisory Boards or participated in continuing medical education programs sponsored by: Shire, Alcobra, Otsuka, McNeil, Janssen, Novartis, Pfizer and Eli Lilly. S. Faraone receives royalties from books published by Guilford Press: Straight Talk about Your Child's Mental Health, Oxford University Press: Schizophrenia: The Facts and Elsevier, ADHD: Non-Pharmacologic Treatments. All other authors report no conflict of interest.

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

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Supplementary Material

IMpACT-NL cohort

Cohort description

All patients were assessed using the Diagnostic Interview for Adult ADHD (DIVA) (Kooij, 2010). This interview focuses on 18 DSM-IV symptoms of ADHD and uses concrete and realistic examples to thoroughly investigate whether a symptom is currently present or was present in childhood. In order to obtain information about ADHD symptoms and impairment in childhood, additional information was acquired from parent and school reports, whenever possible. The Dutch version of the Structured Clinical Interview for DSM-IV (SCID-I and SCID-II) (Groenestijn, 1999; Weertman et al., 2000) was used for lifetime comorbidity assessment. The assessments were carried out by trained professionals (psychiatrists or psychologists). Also, a quantitative measure of clinical symptoms was obtained using the ADHD-DSM-IV Self Rating scale (Kooij et al., 2005). Exclusion criteria for participants were psychosis, alcohol or substance use disorder in the last 6 months, current major depression, full-scale IQ estimate < 70 (estimated from Block Design and Vocabulary of the Wechsler Adult Intelligence Scale-III), neurological disorders, sensorimotor disabilities, non-Caucasian ethnicity, and medication use other than psychostimulants or atomoxetine. An exclusion criteria for healthy control subjects was a current neurological or psychiatric disorder according to SCID-I.

Neuroimaging, MRI acquisition and data processing

Shortly, the images were acquired using a T1.5 MRI scanner (Avanto, Siemens Medical Systems, Erlangen, Germany) at the Donders Centre for Cognitive Neuroscience. All scans covered the entire brain and had a voxel size of 1 x 1 x 1 mm^3, TR 2730 ms, TI 1000 ms, TE 2.95 ms, 176 sagittal slices, field of view 256 mm. To study local differences in gray and white matter related to genetic variation T1 scans were normalized to standard space (Montreal Neurological Institute; MNI; (http://www.mni.mcgill.ca/)), bias corrected, and segmented into gray matter and white matter using the VBM8-toolbox, (http://dbm.neuro.uni-jena.de/vbm/), in SPM8 (http://www.fil.ion.ucl.ac.uk/spm) with default settings. Images were modulated by the non-linear part of their DARTEL warp field (Ashburner, 2007) and smoothed with an 8 mm FWHM Gaussian smoothing kernel. This provided images for an analysis of relative differences in regional grey and white matter volume, corrected for individual brain size. Total volume of gray and white matter was calculated by adding the resulting tissue probability maps. TBV was defined as the voxel-wise sum of white and gray volume.

Genetic data

Genome-wide genotyping of 220,000 single nucleotide polymorphisms (SNPs) was performed at the Lifelines facility (Groningen, The Netherlands). The following quality

control steps were performed for genotype data: SNPs were excluded from analyses if the call rate per SNP was less than 95%, minor allele frequency (MAF) was less than 1%, or if SNPs failed the Hardy-Weinberg equilibrium (HWE) test at a threshold of $p \leq 10^{-6}$ (genome-wide). Participants were excluded from the analysis if the call rate per individual was lower than 95% or if they were genetically related with each other (N=10). To increase genomic coverage, the genotyping data was imputed by using SHAPEIT (Delaneau et al., 2012; Delaneau et al., 2013) (version 1) and IMPUTE2 (Howie et al., 2009) (version 2.2.2). For imputation, the 1000 Genomes haplotypes were used as a reference panel (Phase I integrated variant set release (v3) in NCBI build 37 (hg19) coordinates, downloaded from http://mathgen.stats.ox.ac.uk/impute/data_download_1000G_phase1_integrated.html) (Genomes Project et al., 2010). Average genome-wide concordance rate of genotypes versus imputed genotypes was 95%. Post-imputation quality control included the same control steps as described above for pre-imputation data cleaning. After these steps we had 9,329,570 SNPs across the whole genome. After quality control of MAF ≥ 0.01 and INFO-score ≥ 0.9, 52 SNPs with valid rs-numbers on the *GIT1* locus remained for gene-based analysis (UCSC Genome Bioinformatics Site, http://genome.ucsc.edu/; **Supplementary Table 3**).

Validation of rs550818 genotype by TaqMan genotyping assay

Forward and reverse primer and probes are labeled with a FAM™ and VIC® dye (VIC-GAGGCTGGACCTTGGACTTTAAGCC, FAM-CCAGCTGAGATCAGGGCCCAGTTTG). A total of 0,0625 µl of the *GIT1* rs550818 assay was added to 2,5 µl of Taqman® Universal PCR Mastermix containing a PCR buffer, Amplitaq Gold® DNA polymerase and dNTPs (all Life Technologies). MilliQ was added to adjust the volume to 5 µl. One µl of 10 ng/µl DNA was added to the mix. The PCR protocol included activation of AmpliTaq Gold enzyme for 12 minutes at 95°C, followed by 40 cycles of denaturation of DNA at 92°C for 15 seconds, and annealing and extension of primers at 60°C for 1 minute. As controls, 5-10% duplicates and at least 3-4% blanks were used in the plates.

Gene expression analysis using Taqman assays

The mRNA gene expression analysis of *GIT1* and *GUSB* was carried out in a volume of 10 µl. The PCR mix for one reaction contained 17 ng of RNA in 2.5 µl, 5 µl of 2x Taqman master mix, 0.25 µl of the Taqman assay, and 2.25 µl of purified water. The amplification protocol consisted of an initial step at 50°C for 2 min, followed by a denaturation step at 95°C for 10 minutes, 15 seconds at 95°C, and 1 min at 60°C. The latter two steps were repeated 40 times. PCR was run and the fluorescent signal was measured on an Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies). All Taqman gene expression assays were validated before use, using a 2-fold dilution series and assessing PCR efficiency (slope) and R^2 (*GUSB*: slope=1.1782, R^2=0.9976 and *GIT1*: slope=1.2567, R^2=0.9730).

BIG cohort

Neuroimaging, MRI acquisition and data processing

To study local differences in gray and white matter related to genetic variation, we used the same processing protocol as in the IMpACT study (Hoogman et al., 2014). For a subset of the BIG subjects (N=255), DTI data was acquired in conjunction with the T1-scan, using a standard eddy-current compensated pulsed gradient spin echo - echo planar imaging (PGSE-EPI) (Reese et al., 2003) with some variation in the number of diffusion directions (30-92), b-value (700-1500 s/mm^2) and resolution (2-2.5 mm isotropic).

Images were realigned (affine) and diffusion tensors robustly estimated (PATCH) (Zwiers, 2010). To assess the microstructure of gray and white matter, two diffusion tensor derivatives, i.e. the mean diffusivity (MD) and fractional anisotropy (FA) were computed and normalized to standard space using the T1 DARTEL warp field.

Supplementary Table 1. Description of individual samples

Study name		Study design	Ancestry	Cases (N[a])	Controls (N[a])	Genotyping platform	Reference[b]
Short	Full sample name						
CARD	Cardiff University	Case/ control	Caucasian	641	1,752	Illumina Human660W-Quad BeadChip (ADHD cases) and Illumina Human 1.2M BeadChip (controls)	(Stergiakouli et al., 2012)
CHIN	Peking University	Case/ control	Han Chinese	1,012	930	Affymetrix 6.0 array	(Yang et al., 2013)
GERM	ADHD patient sample consisted of children and adolescents Aachen, Cologne, Essen, Marburg, Regensburg, and Würzburg	Case/ control	Caucasian	494	1,297	Illumina Human660W-Quadv1(ADHD cases) and Illumina HumanHap550v3 (controls)	(Hinney et al., 2011)
IMAGE2	Phase II of IMAGE	Case/ control	Predominantly European origin	787	7,082	Affymetrix 5.0 array (ADHD cases) and Affymetrix 6.0 arrays (controls)	(Neale et al., 2010)
SPAN	Barcelona University	Case/ control	Caucasian	591	432	Illumina HumanOmni1-Quad BeadChip platform	(Sanchez-Mora et al., 2014)
CHOP	Children's Hospital of Philadelphia	Trio	European descent	358		Illumina Infinium II HumanHap550 BeadChip	(Elia et al., 2010)
CROS	Toronto University, SickKids project	Trio	Mainly Caucasian	170		Affymetrix 6.0 array	(Lionel et al., 2011)
IMAGE1	Phase I of the International Multisite ADHD Genetics Project	Trio	Western European origin	866		Perlegen Sciences 600 K	(Neale et al., 2008)
TOTAL				**5,621**	**13,589**		

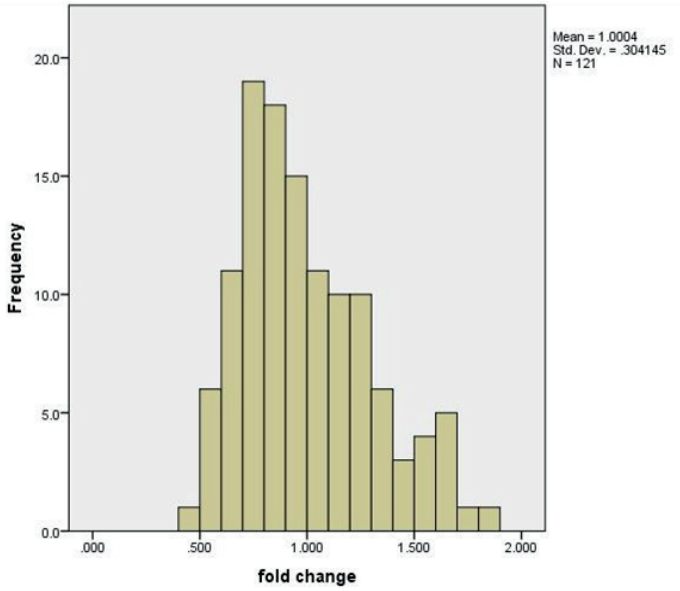
[a] Based on sample of primary publication [b] Primary publication reporting individual study sample.

Supplementary Table 2: Overview of neuropsychological tasks and variables used in this study.

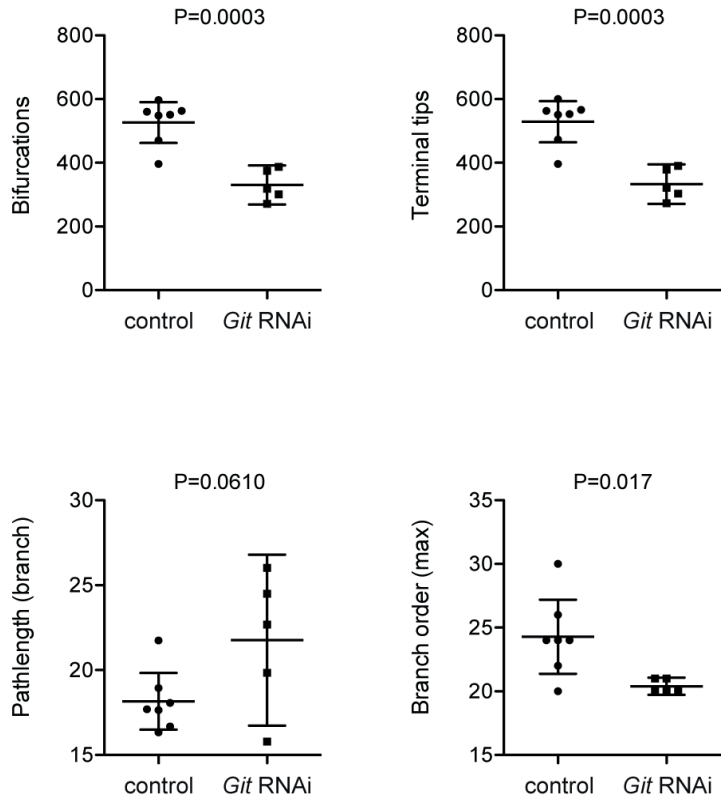
Task	Cognitive domain	Outcome measure used in this study	Reference
Sustained attention dots task (SA-dots)	Attention and inhibition	- Mean series completion time - SD series completion time* - SD series errors (SD of the errors made across blocks) * - Response bias (the difference between the number of misses and the number of false alarms across the entire task).	Part of the ANT testing battery (Huijbregts et al., 2008)
WAIS-III Digit Span task	Working memory	- Forward digit span score (raw) - Backward digit span score (raw)	Subtest of the Wechsler Adult Intelligence Scale (Wechsler, 1997)
Flanker task	Inhibition	- Total SD of RT (average over part 1 and 2)	Part of the ANT testing battery (Huijbregts et al., 2002)
Sustained Attention to Response Task (SART)	Attention and inhibition	- SD of RT hits*	(Smit et al., 2004)
Delay Discounting	Delay aversion and impulsivity	- K 100 (impulsivity high rewards)*	(Dom et al., 2006)
Trail making task	Motor control and set-shifting	- Time to complete part A - Time to complete part B	(Kortte et al., 2002)

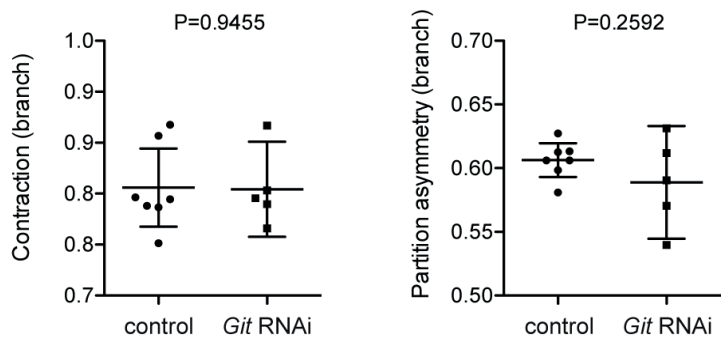
ANT = 'Amsterdamse Neuropsychologische Taken' (De Sonneville, 1999); RT = reaction time; SD = standard deviation; * = variables were log-transformed to guarantee a normal distribution (Mostert et al., submitted).

Supplementary figures



Supplementary Figure 1. Frequency distribution of *GIT1* fold change of 121 IMpACT-NL RNA samples. Distribution meets criteria of normality (Skewness statistic=0.691, standard error=0.220 and Kurtosis statistic=-0.159 and standard error=0.434).





Supplementary Figure 2. Quantification of additional dendrite branching parameters. Quantification of dendrite branching in *Drosophila* class IV da neurons revealed that *Git* RNAi (N=5) altered several dendrite branching parameters, compared to the control (N=7). Scatter dot plots represent individual measurements mean, and error bars indicate the 95% confidence interval. Group differences were assessed by two-tailed Student's or Welch's t-tests.

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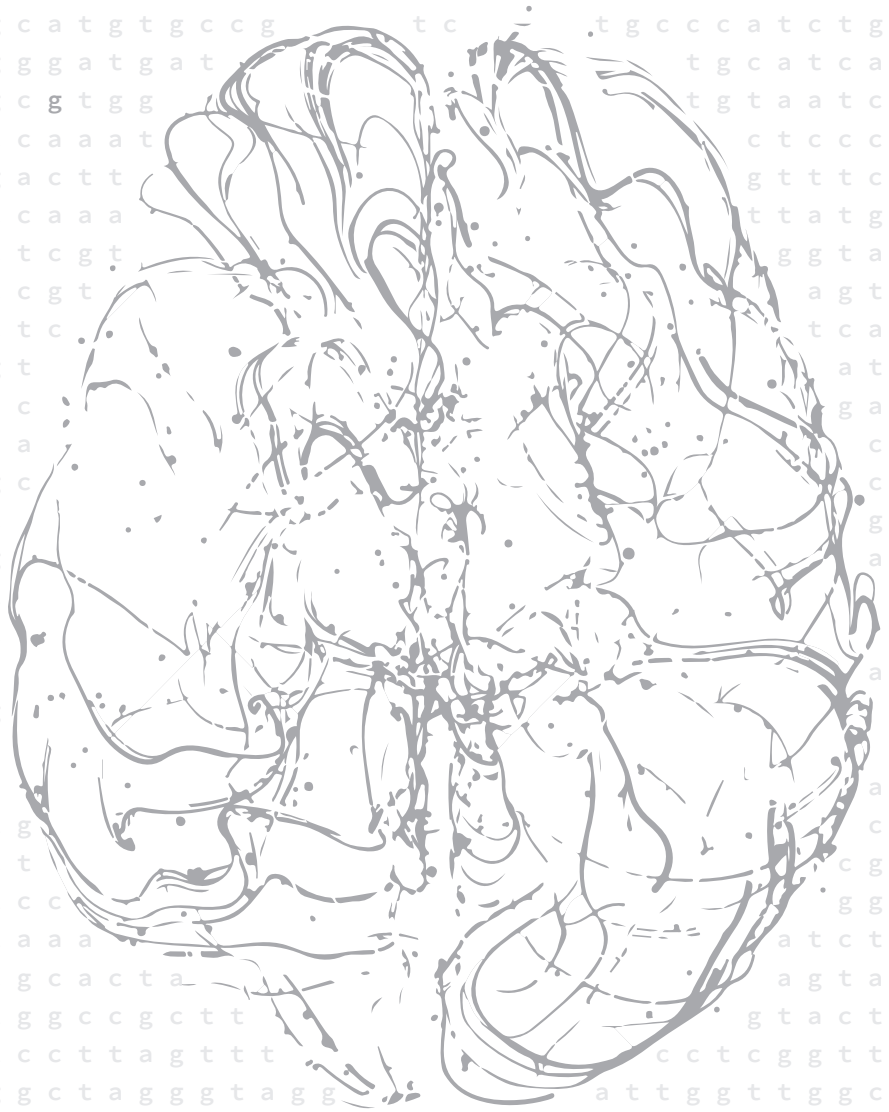

CHAPTER 10

Genetic markers of ADHD-related variations in intracranial volume

Accepted as:
M. Klein, R. K. Walters, D. Demontis, J. L. Stein, D. P. Hibar, H. H. Adams, J. Bralten, N. Roth Mota, R. Schachar, E. Sonuga-Barke, M. Mattheisen, B. M. Neale, P. M. Thompson, S. E. Medland, A. D. Borglum, S. V. Faraone, A. Arias-Vasquez**, and B. Franke** (2018). "Genetic markers of ADHD-related variations in intracranial volume." Am J Psychiatry (also available on bioRxiv; https://doi.org/10.1101/184192).
**shared final responsibility

Abstract

Attention-Deficit/Hyperactivity Disorder (ADHD) is a common and highly heritable neurodevelopmental disorder with a complex pathophysiology. Intracranial volume (ICV) and volumes of nucleus accumbens, amygdala, caudate nucleus, hippocampus, and putamen are smaller in people with ADHD compared to healthy individuals. In this study, we investigated the overlap between common genetic variation associated with ADHD risk and these brain volume measures in order to identify underlying biological processes contributing to the disorder. We combined genome-wide association results from the largest available studies of ADHD (N=55,374) and brain volumes (N=11,221-24,704) using a set of complementary methods to investigate overlap at the level of global common variant genetic architecture and the single variant level. We discovered a negative genetic correlation between ADHD and ICV (r_G=-0.22, p=0.0001543). Meta-analysis of single variants revealed two significant loci of interest associated with both ADHD risk and ICV; four additional loci were identified for ADHD and amygdala, caudate nucleus, and putamen volumes. Exploratory gene-based and gene-set analyses in the ADHD-ICV meta-analytic data showed association with variation in neurite outgrowth-related genes. This is the first genome-wide study to show significant genetic overlap between brain volume measures and ADHD, both on the global and the single variant level. Variants linked to smaller ICV were associated with increased ADHD risk. These findings can help us to develop new hypotheses about biological mechanisms, by which brain structure alterations may be involved in ADHD disease etiology.

Keywords: ADHD, ICV, MRI brain imaging, genetics, neurite outgrowth

Introduction

Attention-Deficit/Hyperactivity Disorder (ADHD) is a common, highly heritable (Faraone et al., 2015; Faraone et al., 2005) neurodevelopmental disorder with a complex and heterogeneous pathophysiology. Pathways towards disease are hypothesized to be mediated by alterations in diverse brain networks (Faraone et al., 2015). A recent neuroimaging mega-analysis reported subtle but consistent differences in volumes of subcortical brain regions and intracranial volume (ICV) in ADHD, across diverse cohorts worldwide: compared to healthy controls, patients with ADHD showed decreased volumes of the nucleus accumbens, amygdala, caudate nucleus, hippocampus, putamen, and ICV (Hoogman et al., 2017). How such alterations contribute to the disease phenotype, is still poorly understood. However, brain volume alterations are also present, on average, in unaffected relatives of patients with ADHD (Bralten et al., 2016; Greven et al., 2015), and both ADHD and brain volumes have high heritability (60-70%(Faraone et al., 2005; Larsson et al., 2014) and 70-90%(Hibar et al., 2015), respectively). This suggests that genetic variants underlying ADHD pathophysiology may also influence brain volume variation. Recently, the first genome-wide significant loci for ADHD were identified and a SNP-based heritability of 20.16% has been reported (Demontis et al., 2017). In the current study, we aimed to investigate the genetic covariance between ADHD risk and structural brain phenotypes; we set out to determine, if common genetic variants are shared between ADHD risk and brain volumes found altered in ADHD (ICV, nucleus accumbens, amygdala, caudate nucleus, hippocampus, putamen). These volumes were selected to focus on the most robust imaging phenotypes in ADHD (Hoogman et al., 2017).

Genome-wide association studies (GWAS) have identified ten genome-wide significant loci associated with hippocampal volume (Bis et al., 2012; Hibar et al., 2017; Hibar et al., 2015; Melville et al., 2012; Stein et al., 2012), eight with ICV (Adams et al., 2016; Hibar et al., 2015; Ikram et al., 2012; Stein et al., 2012), four with putamen volume (Hibar et al., 2015), and one with caudate volume (Hibar et al., 2015). These variants explain only a small fraction of the heritability of these brain volumes (Bis et al., 2012; Hibar et al., 2015; Ikram et al., 2012; Melville et al., 2012; Stein et al., 2012). Recently, Franke and colleagues reported a battery of statistical tools to comprehensively examine genetic overlap between brain volumes and risk for brain disease at the genome-wide level and at the level of individual risk variants, using schizophrenia as an example (Franke et al., 2016). Here, we applied a similar set of methods to identify and dissect genetic sharing between ADHD and brain volumes implicated in ADHD based on the latest mega-analysis (Hoogman et al., 2017).

Material and methods

Ethics statement

This study used summary statistics of GWAS meta-analyses (GWAS-MA) that had been approved by local ethics committees and had required informed consents (described in earlier publications (Adams et al., 2016; Demontis et al., 2017; Hibar et al., 2017; Hibar et al., 2015)).

Participant samples

We used summary statistics data from three consortia (**Supplementary Methods** and **Supplementary Table 1**). GWAS-MA data on ADHD were from the ADHD Working Group of the PGC and the ADHD iPSYCH-SSI-Broad collaboration (n_{cases}=20,183, $n_{controls}$=35,191) (Demontis et al., 2017).

GWAS-MA summary statistics data on ICV and volumes of nucleus accumbens, amygdala, caudate nucleus, hippocampus, and putamen (subcortical volumes were adjusted for ICV to identify specific genetic contributions to individual volumes) were from ENIGMA (Hibar et al., 2015). For the initial GWAS-MA analysis, MRI brain scans and genome-wide genotype data were available for 11,840 subjects. During the reviewing process, we added analyses on the other two subcortical volumes (pallidum and thalamus) for which large-scale GWA data was available (Hibar et al., 2015); results for those can be found in the **extended data sheet** in the **supplementary material**.

Lastly, we obtained summary statistics of additional GWAS-MA data on ICV (n=12,803(Adams et al., 2016)) and hippocampal volume (n=13,039(Hibar et al., 2017)) from the CHARGE Consortium. Importantly, we did not have access to the original or pre-processed MRI scans, but rather used already existing summary statistics data based on initial GWAS-MA that were performed for the different brain volumes of interest.

Prior to all analyses, cohorts including ADHD cases (n=154) were removed from the ENIGMA data (**Supplementary Methods**). The summary statistics data from CHARGE were meta-analyzed with the ENIGMA data sets (**Supplementary Methods**).

To shed some light on the potential role of IQ in the relation between ADHD and brain volume genetics, we also used summary statistics from a GWAS-MA on intelligence performed in 269,867 participants (Savage et al., 2018).

Linkage disequilibrium score regression (LDSR)

GWAS-MA data sets underwent additional filtering (**Supplementary Methods**). The ADHD analysis included only results from studies with samples of European (Caucasian) genetic background (n_{cases}=19,099 and $n_{controls}$=34,194). For the ENIGMA amygdala results, the mean χ^2 was too low (1.0) to reliably estimate SNP heritability using LDSR. **Table 1** shows genetic correlations between brain volumes.

The analysis used a two-step procedure with the LD-scoring analysis package (Bulik-Sullivan et al., 2015). An unconstrained regression estimated regression intercepts for each phenotype. Since we took measures to exclude sample overlap, we also performed the analysis with regression intercept defined as zero (**Supplementary Table 2**). To compute p-values, standard errors were estimated using a block jackknife procedure.

Table 1. Genetic correlations between brain volumes[1]

Trait	Intracranial volume[#]		Nucleus accumbens		Caudate nucleus		Hippocampus[#]		Putamen	
	r_g	P	r_g	P	r_g	P	r_g	P	r_g	P
Intracranial volume[#]	---	---	---	---	---	---	---	---	---	---
Nucleus accumbens	0.02727	0.8353	---	---	---	---	---	---	---	---
Caudate nucleus	0.1158	0.2323	0.4191	0.007684	---	---	---	---	---	---
Hippocampus[#]	0.2559	**0.001704**	0.1891	0.2218	-1.725	0.1991	---	---	---	---
Putamen	0.08746	0.306	0.4357	**$1.67x10^{-3}$**	0.175	0.173	0.08832	0.3802	---	---

[1]Amygdala mean χ^2 was too small to allow a valid analysis (N=11,757). rg=genetic correlation. [#]Using GWAS-MA summary statistics from the meta-analysis of ENIGMA and CHARGE cohorts. Genetic correlations were estimated by using free intercepts. In accordance with the number of tests performed, we set a Bonferroni-corrected significance level at P=0.05/10=0.005. P-values in bold are significant after Bonferroni correction.

SNP effect concordance analysis (SECA)

Post-processing of genetic data

To statistically compare ADHD and six brain volume GWAS-MAs, we used SNPs that passed quality control and filtering rules in all data sets (**Supplementary Methods**). Clumping procedure in PLINK (Purcell et al., 2007) identified an independent SNP from every LD-block across the genome, providing independent SNP sets representing the total variation explained across the genome conditioned on the significance in each brain volume GWAS-MA (**Supplementary Methods**). For each of these SNP sets, we determined the corresponding ADHD GWAS-MA test statistic for each independent index SNP and used these data sets for subsequent analyses.

Tests of pleiotropy and concordance

We used SNP Effect Concordance Analysis (SECA)(Nyholt, 2014) to determine the extent and directionality of genetic overlap between ADHD and each brain volume. Within SECA, we performed a global test of pleiotropy using a binomial test at 12 p-value levels (**Supplementary Methods**). Similarly, a two-sided Fisher's exact test estimated concordance, the agreement in SNP effect directions across two traits. We determined whether there was a significant (P≤0.05) positive or negative trend in the effect of the overlapping SNPs at each p-value threshold (**Supplementary Methods**). In total, we tested for pleiotropy and

concordance between ADHD and six brain volumes. The Bonferroni-corrected significance level was set at P=0.05/(2*6)=4.17x10^{-3}.

SNP sign test in the top GWAS-MA findings

To investigate a potential accumulation of same- or opposite-direction effects of SNPs between ADHD and brain volumes, we counted the number of *opposite* direction effects (as expected from the imaging results in (Hoogman et al., 2017)) for top-findings from the ADHD data set in the different brain structure data sets. The ADHD GWAS-MA data were clumped to define independent loci (**Supplementary Methods**) for all variants with P<1x10^{-5} in the ADHD GWAS-MA using 1KGP3v5(The 1000 Genomes Project Consortium, 2015) data on European ancestry populations as reference.

The proportion of variants with a discordant direction of effect in the individual brain GWAS-MA was evaluated using a binomial test against a null hypothesis of 0.5 (i.e. chance level). This test was done for loci passing p-value thresholds of P<5x10^{-8} (14 LD-independent genome-wide significant SNPs), P<1x10^{-6} (44 LD-independent SNPs), and P<1x10^{-5} (132 LD-independent SNPs) in the ADHD GWAS-MA. Details on the sign tests in the intelligence GWAS-MA data (Savage et al., 2018) are described in the **Supplementary Methods**.

Weighted meta-analysis of ADHD and brain volume data sets

Independent of the results of the global overlap analyses, we also performed meta-analyses combining results from the ADHD GWAS-MA with results from brain volume GWAS-MAs. We used a modified sample size-based weighing method, integrating the binary ADHD trait (ADHD risk) with the continuous trait (brain volume traits), as described in (Demontis et al., 2017). The modified sample size-based weights were derived to account for the respective heritabilities, genetic correlation, and measurement scale of the GWAS-MAs (**Supplementary Methods** and article (Demontis et al., 2017)). For all brain volumes, we additionally performed naive meta-analyses given their low genetic correlations with ADHD risk. We set the threshold for genome-wide significance at P=5x10^{-8}/6=8.33x10^{-9}.

Gene-based and gene-set analyses for ADHD+brain GWAS-MA data

Genome-wide summary statistics of ADHD, individual brain, and weighted meta-analysis ADHD+brain volume GWAS-MA data sets were used as input for gene-based analyses, using the Multi-marker Analysis of GenoMic Annotation (MAGMA) software package (version 1.05,(de Leeuw et al., 2015), **Supplementary Methods**). For the ADHD+brain GWAS-MA, only SNPs shared between ADHD and brain volume data sets were included. Overlapping significant genes (P<2.731x10^{-6}) were determined and selected for further investigation (**Supplementary Methods**).

For gene-set analyses, we used self-contained and competitive testing and tested, whether genes in the neurite-outgrowth gene-set (defined previously, N_{genes}=45;

Supplementary Methods (Poelmans et al., 2011b)) were jointly associated with results of the weighted meta-analytic data of ADHD+ICV (**Supplementary Methods**). *Post-hoc*, individual genes in the set were investigated, by reviewing gene test-statistics of the weighted ADHD+ICV GWAS-MA results. Genes reaching Bonferroni correction threshold (P=0.05/45=0.00111) were considered gene-wide significant. Subsequently, we reviewed gene-based associations in the ADHD GWAS-MA and ENIGMA+CHARGE ICV GWAS-MA results separately.

Reciprocal lookup of significant GWAS-MA loci

Evidence for an effect of ADHD-associated SNPs on brain volume was studied through a look-up of results in the ENIGMA (+CHARGE) GWAS-MAs. LD-independent loci with corresponding index SNPs were obtained by clumping the summary statistics of the ADHD GWAS-MA (Demontis et al., 2017) (**Supplementary Methods**). Similarly, effects of 21 independent SNPs significantly associated with brain volumes in the original publications of the brain volume GWAS-MAs (Adams et al., 2016; Hibar et al., 2017; Hibar et al., 2015) on ADHD risk were looked-up in the ADHD GWAS-MA data. If the index variant was not present in the other data set, a proxy variant was selected through LDlink (https://analysistools.nci.nih.gov/LDlink/). The Bonferroni-corrected significance levels were set at P=0.05/(14*8)=0.000446 for look-up of ADHD SNPs in brain volume GWAS-MAs and at P=0.05/21=0.002381 for brain volume SNPs in ADHD GWAS-MA data.

Expression quantitative trait loci and brain gene expression

Expression quantitative trait loci (eQTL) were examined using data from the GTEx portal (https://www.gtexportal.org/home/)(GTEx Consortium, 2013) and the Blood eQTL Browser (http://genenetwork.nl/bloodeqtlbrowser/)(Westra et al., 2013).

We investigated the spatio-temporal expression pattern in brain tissue for selected genes using data from the Human Brain Transcriptome Project (http://hbatlas.org). We assessed messenger RNA (mRNA) expression trajectories in six regions of the developing and adult human brain (**Supplementary Methods**). Gene expression over the lifespan from the spatio-temporal atlas was graphed using custom R scripts (Kang et al., 2011).

Results

Comparison of common variant genetic architectures

Linkage disequilibrium score regression. SNP-based heritability estimates for the MRI measures were consistent with prior reports (Adams et al., 2016; Franke et al., 2016; Hibar et al., 2017) and ranged from 13.32% to 28.15% (**Table 2**). The amygdala mean χ^2 was too small to allow a valid analysis. We observed a significant negative genetic correlation between

ADHD and ICV (rg=-0.227, P=0.00015). All other correlations were non-significant (**Table 2**; **Supplementary Table 2** shows results when using constrained intercepts).

Table 2. SNP heritability analyses for MRI brain volumes and genetic correlation with ADHD[1].

Brain region	N	Herita-bility	SE	Genetic correlation with ADHD	SE	Z	P
Nucleus accumbens	11,709	0.1332	0.0518	0.005558	0.09487	0.05858	0.9533
Caudate nucleus	11,772	0.2456	0.0455	-0.06426	0.07321	-0.8778	0.3801
Hippocampus[2]	24,704	0.1418	0.0286	-0.02354	0.06902	-0.3411	0.733
Intracranial volume[2]	24,024	0.2318	0.0325	-0.2266	0.05989	-3.784	**0.0001543**
Putamen	11,646	0.2815	0.056	0.006433	0.07077	0.09089	0.9276
Hippocampus ENIGMA only	11,665	0.1363	0.0488	-0.04202	0.09965	-0.4217	0.6733
Hippocampus CHARGE only	13,039	0.16	0.042	-0.0126	0.0832	-0.1516	0.8795
Intracranial volume ENIGMA only	11,221	0.1745	0.0461	-0.2348	0.09452	-2.485	0.01296
Intracranial volume CHARGE only	12,803	0.283	0.0466	-0.2305	0.071	-3.2479	0.0012

[1]Amygdala mean χ^2 was too small to allow a valid analysis (N=11,757). [2] Using GWAS-MA summary statistics from the meta-analysis of ENIGMA and CHARGE cohorts. Heritability and genetic correlation were estimated by using free intercepts. In accordance with the number of tests performed, we set a Bonferroni-corrected significance level at P=0.05/6=0.0083. P-values in bold are significant after Bonferroni correction.

SNP effect concordance analysis. SECA found significant evidence of global pleiotropy for variants affecting ADHD risk for volumes of four subcortical brain regions and ICV (**Table 3** and **Supplementary Fig. 1**). Discordant SNP effects for ADHD and ICV were significant, i.e. variants increasing the risk for ADHD were associated with decreased ICV (P_{ICV}<0.001; **Table 3** and **Supplementary Fig. 2**). Evidence for concordant SNP-effects reached significance for ADHD and nucleus accumbens ($P_{accumbens}$=0.002), and for ADHD and caudate nucleus ($P_{caudate}$=0.004, **Table 3** and **Supplementary Fig. 2**).

Sign tests. Based on the phenotypic observation that patients with ADHD have, on average, smaller brain volumes compared to healthy controls (Hoogman et al., 2017), we had expected discordant rather than concordant SNP effects. As both discordant and concordant effects were seen in the SECA, we specifically determined directionality of genetic overlap between ADHD and brain volume for the top-associations per trait. Thus, we zoomed in further on the most strongly associated and LD-independent SNPs and compared the signs of the regression coefficients of those top-associations per trait. None of the sign tests showed a consistent direction of discordance, after correction for multiple testing (**Supplementary Table 3**). Additionally, LD-independent ADHD+ICV-associated SNPs

showed an overrepresentation of discordant effects in GWAS-MA data for intelligence (30 out of 43 SNPs, proportion=0.698, P=0.0069; **Supplementary Table 4**(Savage et al., 2018)).

Table 3. Results of pleiotropy and concordance test of SNP Effect Concordance Analysis. Brain volume GWAS-MA was conditioned on ADHD GWAS-MA.

Brain volume	$P_{pleiotropy}$	$CI_{pleiotropy}$	$P_{concordance}$	$CI_{concordance}$	Direction of SNP effects
Nucleus accumbens	0.034	0.0244-0.0471	**0.002**	0.000548-0.00726	concordant
Amygdala	**<0.001**	$5.12x10^{-5}$-0.00564	0.006	0.00275-0.013	discordant
Caudate nucleus	**<0.001**	$5.12x10^{-5}$-0.00564	**0.004**	0.00156-0.0102	concordant
Hippocampus[1]	**0.002**	0.000548-0.00726	1	0.996-1	/
Intracranial volume[1]	**<0.001**	$5.12x10^{-5}$-0.00564	**<0.001**	$5.12x10^{-5}$-0.00564	discordant
Putamen	**<0.001**	$5.12x10^{-5}$-0.00564	0.01	0.00544-0.0183	concordant
Hippocampus ENIGMA only	0.005	0.00214-0.0116	1	0.996-1	/
Intracranial volume ENIGMA only	**<0.001**	$5.12x10^{-5}$-0.00564	**<0.001**	$5.12x10^{-5}$-0.00564	discordant

P-values and confidence intervals (CI) were obtained based on 1,000 permutations. [1] Using GWAS-MA summary statistics from the meta-analysis of ENIGMA and CHARGE cohorts. In accordance with the number of tests performed, we set a Bonferroni-corrected significance level at P=0.05/(2*6)=0.00416. P-values in bold are significant after Bonferroni correction.

Analyses at the single genetic variant level

Weighted SNP meta-analyses. Based on the findings of both concordant and discordant links between ADHD and the brain volume SNPs, we performed a genome-wide search for specific genetic loci associated with both ADHD and each brain trait. We used a weighted SNP meta-analysis design allowing the combination of findings from GWAS of binary and quantitative variables (Demontis et al., 2017), enabling us to specifically look for concordant effects at the level of single genetic variants; there is currently no suitable method to study discordant effects. The weighted GWAS-MA for ADHD and ICV identified two significant loci of interest: chromosome 15 (*SEMA6D*) and chromosome 16 (*intergenic*; **Table 4**, **Figures 1** and **2**). Four additional loci passed the study-wide threshold for genome-wide significance, but those were related to a single phenotype and did not meet criteria for cross-trait relevance (**Figure 1**).

We also performed weighted GWAS-MAs for ADHD and the four subcortical brain structures (**Supplementary Figures 3-7**). For amygdala volume, a naïve sample size-weighted meta-analysis was performed, as no genetic correlation with ADHD had been estimated; the six novel and/or improved LD-independent genome-wide significant loci observed in these analyses are summarized in **Table 4.** Among those, the *SEMA6D* locus was significantly associated with ADHD and putamen volume (P=$3.62x10^{-9}$; **Table 4, Figure 2**, and **Supplementary Fig. 7**).

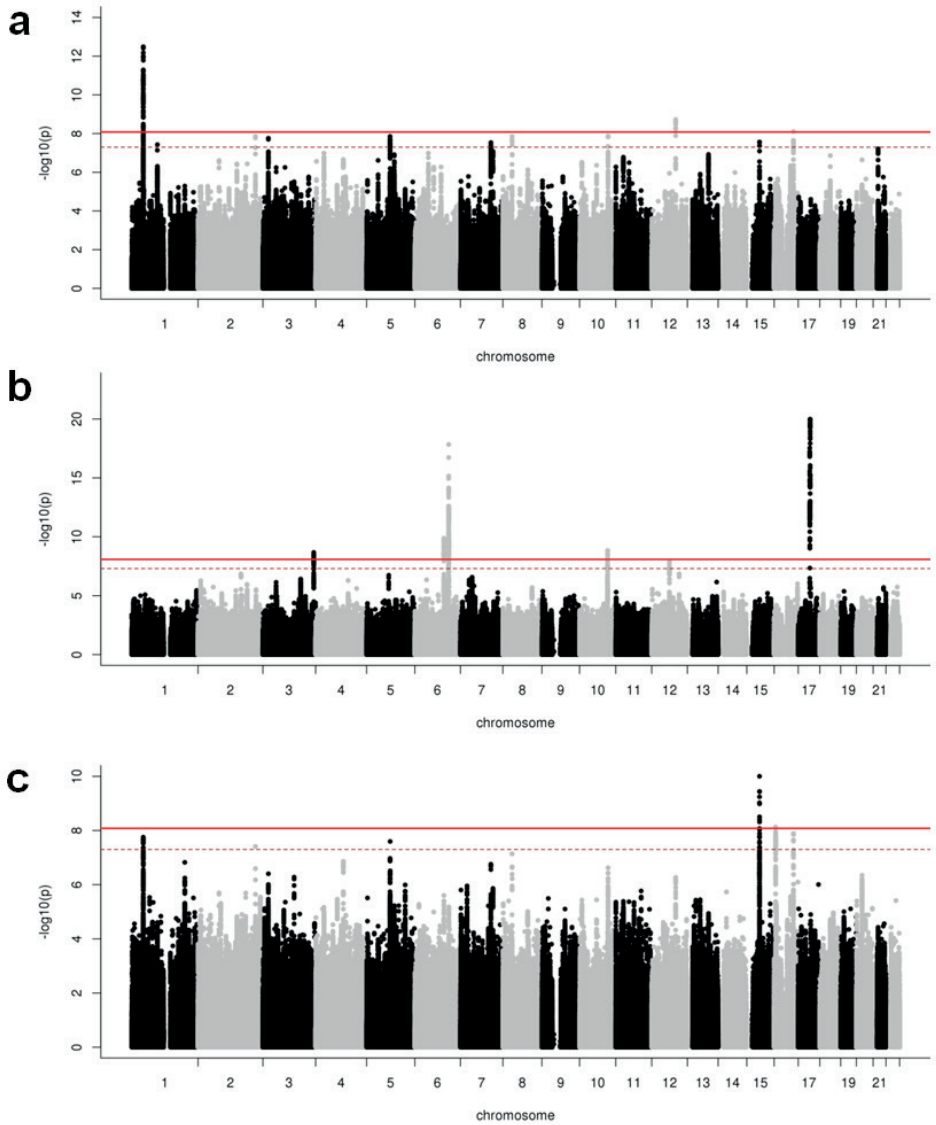

Figure 1: Common genetic variants associated with ADHD, ICV and ADHD+ICV. Shown here are Manhattan plots, in which every point represents a single genetic variant plotted according to its genomics position (x-axis) and its $-\log_{10}(P)$ for association with the respective trait (y-axis). The solid bright red line represents the study-wide genome-wide significance of $P<8.33x10^{-9}$, and the dashed dark red line represents the genome-wide significance of $P<5x10^{-8}$. (**a**) PGC+iPSYCH ADHD GWAS-MA. (**b**) ENIGMA+CHARGE ICV GWAS-MA. (**c**) ADHD+ICV weighted GWAS-MA.

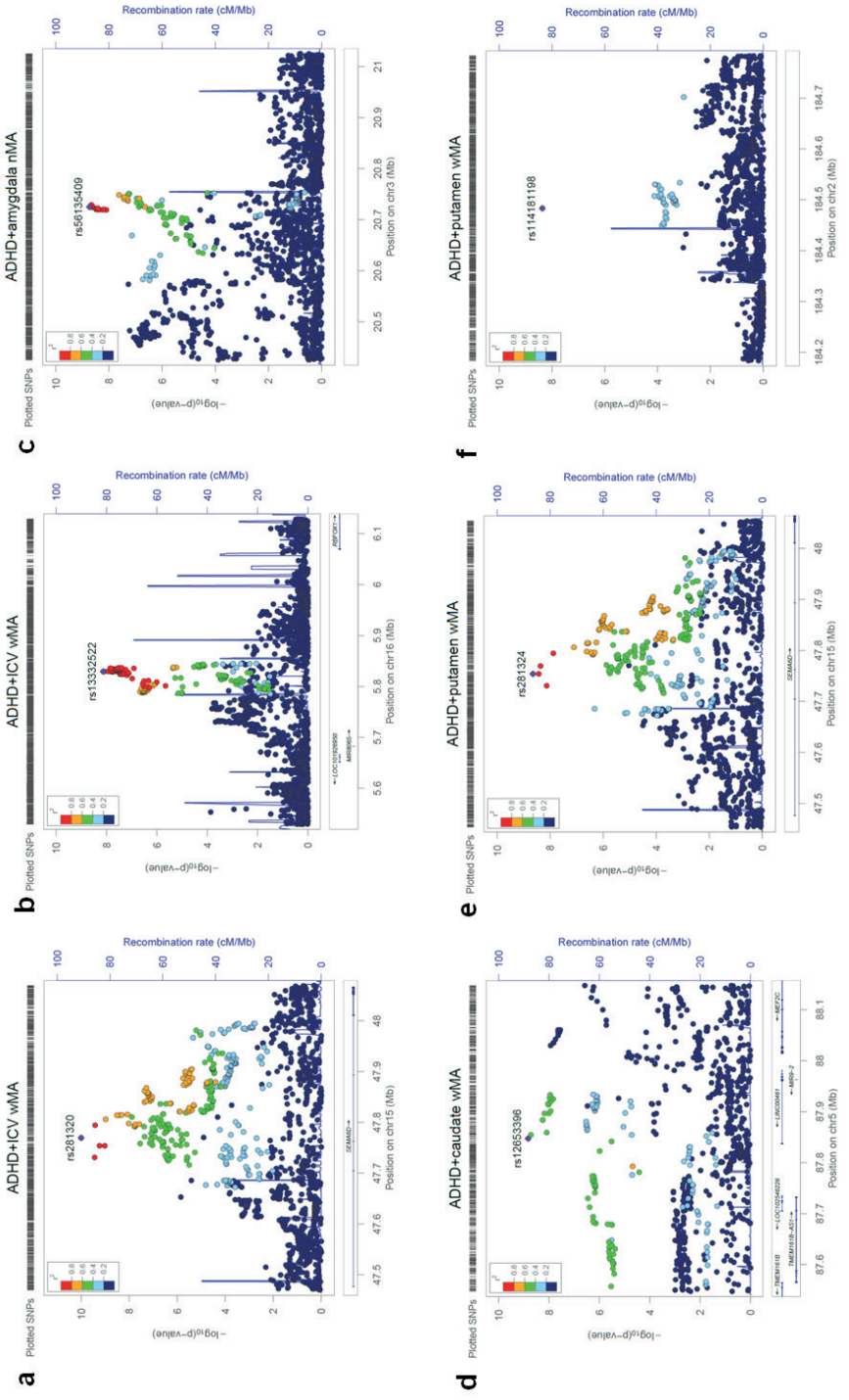

Figure 2: Regional association of genome-wide significant loci of ADHD and brain volume GWAS-MAs. For each panel (**a-f**), zoomed-in association plots (± 300 kb from the top SNP, indexed by purple dot) are shown. Plots are zoomed to highlight the genomic region that likely harbors the causal variant (s).

Table 4: Results of (weighted) meta-analyses of ADHD and brain volumes showing novel or improved independent genome-wide significant loci.

Brain trait	Chr	Pos	Gene	SNP	A1	Zscore	P-value	Direction ADHD / brain	$Zscore_{ADHD}$	OR_{ADHD}	$Zscore_{brain}$	$Effect\ (se)_{ENIGMA2}$	$Variance\ explained_{ENIGMA2}$ (%)
Intracranial volume	15	47769424	*SEMA6D*	rs281320[1]	T	-6.4677	$9.95x10^{-11}$	-/-	-5.5487	0.9252	-3.33	-4772.06 (1923.4439)	0.0548
Intracranial volume	16	5829204	intergenic	rs13332522[2]	C	5.7810	$7.43x10^{-9}$	+/+	4.6162	1.0678	3.508	2986.546 (2174.0614)	0.0168
Amygdala	3	20725016	intergenic	rs56135409[1]	A	-6.0105	$1.85x10^{-9}$	-/-	-5.3525	0.9268	-2.7482	-6.8605 (2.4964)	0.0653
Caudate nucleus	5	87847273	*LINC00461*	rs12653396[2]	A	5.3826	$1.46\ x10^{-9}$	+/+	5.3777	1.0757	2.9499	14.8669 (5.0397)	0.0745
Putamen	15	47754027	*SEMA6D*	rs281323[1]	A	5.9005	$3.62\ x10^{-9}$	+/+	5.4775	1.0811	2.2114	13.6151 (6.1567)	0.0420
Putamen	2	184483159	intergenic	rs114181198[2]	A	5.1308	$4.28\ x10^{-9}$	+/+	5.0859	1.1259	3.2596	36.067 (11.0649)	0.0927

Results for six genome-wide significant index SNPs identified in the GWAS meta-analyses of ADHD+brain volumes. The allele (A1) for the effect of the Z-score is given. Direction gives information about the direction of the association in the two samples, "+" indicates that A1 increases risk/volume and "-" indicates that A1 decreases risk/volume. The threshold for genome-wide significance was set at $P<8.33x10^{-9}$ ($5x10^{-8}/6$ traits). [1] This locus improved by one order of magnitude after the meta-analysis. [2] This locus is a new genome-wide significant locus in the ADHD+brain volume meta-analysis. OR_{ADHD} is based on initial PGC+iPSYCH GWAS-MA that was used as input for the weighted meta-analysis. Effect sizes are given in units of mm3 per effect allele. Results are provided for the ENIGMA2 cohort only. The variance explained gives the percentage variance explained by a given SNP after correcting for covariates (see **Supplementary Methods** for additional details).

Gene-wide GWAS-MAs. To maximize power of the meta-analysis, we ran genome-wide gene-based GWAS-MAs in MAGMA; for gene-based results of all genes see **Supplementary Tables 5-10**. For ADHD+ICV, three genome-wide significant genes (*MEF2C, KIZ*, and *SEMA6D*) showed stronger association in the cross-trait meta-analysis compared to the separate analyses of ADHD and ICV (**Supplementary Table 11**). Additionally, the genome-wide significant genes *FEZF1* (amygdala), *ADD1* (caudate nucleus), and *MANBA* (hippocampus) showed increased significance in the cross-trait meta-analyses, compared to the individual analyses of ADHD and brain volumes (**Supplementary Tables 12-16**).

Reciprocal lookup of genome-wide significant associations. No significant associations were observed between the 14 previously identified genome-wide significant ADHD SNPs (Demontis et al., 2017) and brain volumes (**Supplementary Table 17**). Conversely, among 21 SNPs previously associated with the brain volumes (Adams et al., 2016; Hibar et al., 2017; Hibar et al., 2015), association of two ICV-linked variants (rs8756 and rs2195243) with ADHD survived correction for multiple testing (**Supplementary Table 18**; $P<0.00238$).

Expression quantitative loci and brain gene expression

Previously, it was shown that many SNPs in the *SEMA6D* locus were strongly associated with expression of *SEMA6D* in fibroblasts (Demontis et al., 2017; GTEx Consortium, 2013). Indeed, repeating this analysis for the most strongly associated variants identified by the (weighted) cross-phenotype SNP meta-analyses and the two significant variants of the reciprocal lookup using the GTEx data (GTEx Consortium, 2013), we found rs281320 to be a significant eQTL for *SEMA6D* in transformed fibroblast tissue ($P=1.2x10^{-20}$; **Supplementary Table 19**), as was rs281323 ($P=1.2x10^{-21}$). The alternative alleles of both rs281320 and rs281323, which are associated with increased risk for ADHD and larger ICV, also increased *SEMA6D* expression (**Supplementary Fig. 9, a** and **b**). Additionally, rs12653396 was a significant eQTL for the *CTC-498M16.4* and *MEF2C* genes, in brain ($P=2x10^{-7}$)(GTEx Consortium, 2013) and blood tissue ($P=6.53x10^{-7}$)(Westra et al., 2013), respectively, with the disease-associated A-allele being associated with increased *MEF2C* expression (**Supplementary Table 19**). Both rs8756 and rs2195243 were eQTLs of *HMGA2* and *CCDC53*, respectively. All other top-SNPs were not present in either of the two eQTL databases.

We determined mRNA expression for *SEMA6D* and *MEF2C*, the only protein-coding genes identified in the SNP-based cross-phenotype GWAS-MAs and four significant genes (*FEZF1, ADD1, MANBA, KIZ*) identified in the gene-based cross-trait analyses, in six brain regions of the developing and adult human brain using data from the Human Brain Transcriptome Project (Kang et al., 2011). All genes are globally expressed in the developing and adult human brain, with *SEMA6D* and *MEF2C* showing highest mRNA expression in prenatal periods (**Supplementary Fig. 8**).

Neurite outgrowth gene-set analysis

In an exploratory analysis, we found an association between a pre-defined gene-set of 45 neurite outgrowth genes (Poelmans et al., 2011b) and the meta-analytic data for ADHD+ICV using MAGMA ($P_{competitive}$=0.00338). It is current practice to use competitive tests, for completeness, we also report results from the self-contained analysis, which was $P_{self\text{-}contained}$=1.55x10^{-6}. Associations of this set with ADHD, separately, were restricted to the self-contained test ($P_{self\text{-}contained}$=5.53x10^{-9}); for ICV, no significant associations with the gene-set were found (**Supplementary Table 20**). In the ADHD+ICV GWAS-MA, the most strongly associated individual neurite outgrowth gene in the set was *CREB5* (P=0.000553); nine additional neurite outgrowth genes showed nominally significant associations (**Supplementary Table 21**; for gene-based results of all genes see **Supplementary Table 10**).

Discussion

In this study we set out to investigate genetic covariance between ADHD risk and structural brain phenotypes. We found significant - though modest - genetic covariation between ADHD risk and brain volumes, both on global and gene-wide/single variant levels. On the global level, significant negative genetic correlation between ADHD and ICV was demonstrated. The direction of effect was supported by SNP effect concordance analysis. Our ICV finding was highly consistent across approaches and in the expected direction, given the previous observation that patients with ADHD have smaller ICV relative to controls (Hoogman et al., 2017). For most subcortical brain volumes, pleiotropic effects were also found. On the single variant and gene-wide levels, meta-analyses found significant loci associated with both ADHD risk and brain volumes. We identified *SEMA6D, KIZ, and MEF2C* as potential key-loci contributing to both ADHD risk and ICV, and exploratory gene-set analysis revealed association of ADHD-ICV overlap with variation in neurite outgrowth genes.

A reduction of subcortical brain volumes and ICV is not unique to an ADHD diagnosis, but is also seen in depression and bipolar disorder (Hibar et al., 2016; Schmaal et al., 2016). However, genetic correlation between ADHD and ICV shows some specificity to this disorder, as it was not found in studies of other mental disorders, i.e. schizophrenia (Adams et al., 2016; Franke et al., 2016), major depressive disorder (Wigmore et al., 2016), or autism (Adams et al., 2016), using similar methods. On the other hand, power issues should not yet be discarded as a reason for the lack of finding genetic correlations, despite the large sample sizes, and results of a recent study found that schizophrenia and brain structure volumes share genetic risk factors using a conditional false discovery rate (FDR) analysis (Smeland et al., 2018).We observed significant pleiotropy also between ADHD and amygdala, caudate nucleus, hippocampus, and putamen volumes. This global genetic covariation

was substantiated by local effects, which we observed in the weighted cross-phenotype meta-analyses. In addition to ICV, caudate nucleus, and putamen volumes variation also showed significant genetic concordance with ADHD. However, whereas results for ICV were in line with our expectation, concordant effects for ADHD and nucleus accumbens and caudate nucleus volume are counterintuitive (ADHD patients have smaller volumes for these structures (Hoogman et al., 2017)), suggesting a reverse or more complex pattern of causation. Importantly, the subcortical regions were corrected for ICV phenotypically, so that their genetic correlation was limited.

On the single variant level, we only had tools available to perform a meta-analysis by looking at concordant effects (Demontis et al., 2017), we therefore had to ignore locus-specific discordant effects. Still, the strongest association of single genetic markers was observed for ADHD and ICV, additional associations were identified for ADHD and subcortical volumes. The weighted meta-analysis of ADHD and ICV found two potentially pleiotropic loci. One of those was *SEMA6D*, coding for the semaphorin 6D, a transmembrane molecule important for maintenance and remodeling of neuronal connections (Qu et al., 2002). Animal studies showed that it acts as ligand for PlexinA1, which is involved in neuronal development in the spinal cord (Yoshida et al., 2006). Together with the gene-based cross-trait result identifying the *MEF2C* gene and the findings in the exploratory gene-set analysis, our findings suggest that neurite outgrowth dysregulation may act as a neural mediator of ADHD. Dysregulation of neurite outgrowth may pose a more general genetic risk for psychopathology, as it has been shown to not only be involved in ADHD (Mooney et al., 2016; Poelmans et al., 2011b) and the hyperactive/impulsive symptom domain of ADHD (Bralten et al., 2013), but also in dyslexia (Poelmans et al., 2011a) and autism (Poelmans et al., 2013). We also found the *SEMA6D* locus in the cross-phenotype meta-analysis for ADHD and putamen volume, even though this volume had been corrected for ICV, suggesting that genetic variation in *SEMA6D* may influence specific brain regions to a different extent. In line with our gene-set association results, a recent study using data from the UK Biobank mainly found associations between MRI measures and genes involved in brain development and plasticity (Elliott et al., 2017). Since most of these genes have also been demonstrated to contribute to different psychiatric and neurodegenerative disorders (Elliott et al., 2017), specificity of our findings for ADHD requires additional investigation.

The current results raise a number of questions concerning the way that alterations in the brain mediate etiological risk pathways in ADHD. The first question is about the role of cognitive performance in this relationship. ADHD and ICV have recently shown to be genetically correlated with intelligence (ADHD is negatively genetically correlated with IQ (rg=-0.37, p=2.21x10^{-2}), and ICV is positively correlated with IQ (Sniekers et al., 2017) (rg=0.29, p=3.44x10^{-4}). Similarly, educational attainment is linked to both ICV and ADHD (rg=0.34, p=1.2×10^{-6}; rg=-0.54, p=1.44x10^{-80}), as well as to IQ (Demontis et al., 2017; Okbay et al., 2016). It may therefore be possible, that the genetic link between ADHD

and ICV is mediated by IQ and its proxies. We attempted to test this - in the absence of IQ/educational attainment data to correct for in the ADHD GWAS-MA - using a sign test based on data from the recent large intelligence GWAS-MA (Savage et al., 2018). Here, we found an overrepresentation of opposite direction effects of ADHD-ICV SNPs in the intelligence GWAS-MA data, suggesting that intelligence may indeed play a role in the ADHD-ICV overlap. However, out of the 43 SNPs included in the analysis, only 15 (34.8%) were nominally significant associated with intelligence, suggesting that the genetic link between ADHD and ICV is additionally driven by intelligence-independent effects. More in depth research will be needed to fully understand the role of intelligence in ADHD-ICV overlap in the future; it may occur upstream and/or downstream of our correlation finding. Secondly, the degree of sharing observed was statistically modest. At first sight, this seems to be inconsistent with the general hypothesis that ADHD is a genetic-based brain disorder. However, there are a number of possible explanations for this modest sharing. We examined brain structure at a gross anatomical scale: compared to more precise methods, such as voxel-based or surface-based morphometry, atlas-based brain segmentations might be too coarse to identify subtler volumetric differences. Importantly, for the type of imaging genetics analyses described in here, we were strongly dependent on the availability of GWAS data for brain phenotypes. These GWAS data have to be derived from large-scale studies in order to allow sufficiently powered analyses. Such data is so far available only for subcortical volumes and ICV, published by the ENIGMA and CHARGE consortia. The sample sizes of the few voxel-wise GWASs available to date are not large enough to offer sufficient statistical power for the genome-wide approaches presented here. Moreover, it may be more informative to study structural and functional connectivity measures. In addition, as pointed out previously (Franke et al., 2016), the limited SNP-heritability of (subcortical) brain volumes further challenges the identification of genetic overlap, and more highly powered studies of brain phenotypes may lead to higher estimates of overlap. Also, the field may advance by applying more sophisticated imaging approaches to imaging genetics studies, such as redefining imaging phenotypes through dimension reduction approaches (Fan et al., 2018). Finally, it is also possible that some of ADHD's association with reduced brain volumes is driven by environmental effects, either independently or in interaction with genetic factors (Faraone et al., 2015).

Previous brain imaging genetics studies in ADHD mainly focused on single genetic variants and were hampered by limited sample sizes (Klein et al., 2017). This study combined the largest data sets available to investigate the genetic overlap between ADHD and brain volumes by using a complementary battery of statistical methods. Nevertheless, some limitations apply. Firstly, this study focused on a limited set of mainly subcortical MRI measures, and future work should be extended to cortical regions and connectivity measures, once large-scale GWAS-MA becomes available (Bralten et al., 2016; Wu et al., 2017). To support highly sophisticated imaging genetics analyses, which can provide granular

information on specific circuits of relevance, there is also an increasing need for large-scale imaging cohorts with access to raw imaging and genetic data that allow maximal flexibility in the application of analysis methods. Secondly, for the cross-phenotype GWAS-MA, we used a recently described weighted meta-analysis method (Demontis et al., 2017). However, we observed that with low and moderate genetically correlated phenotypes, the association signals did generally not improve over a naïve meta-analysis, performed without adding additional weights (**Supplementary Fig. 10** and **Supplementary Table 22**). In addition, we could only investigate concordant SNP effects with this method. Thirdly, generalization of our findings to other ethnicities should be assessed in future studies. Fourthly, it is possible that this study underestimated genetic correlations, as we did not take into account the known role of rare and structural variants in the genetic architecture of ADHD (Demontis et al., 2016; Williams et al., 2010). Future studies investigating heritability and genetic correlation could also benefit from including variants with low minor allele frequency and in low-LD regions, which may reveal stronger relationships between ADHD and brain volumes.

This is the first study to show significant global and single gene/variant level genetic correlations derived from polygenic overlap between ADHD and brain volumes. The modest genetic overlap between ADHD and variation in brain volumes is consistent with models implicating alterations in brain structure in ADHD-related genetic risk pathways and provides new hypotheses about neuro-biological mechanisms involved in ADHD.

Acknowledgments

Funding: This work was supported by the Netherlands Organization for Scientific Research (NWO), i.e. the NWO Brain & Cognition Excellence Program (grant 433-09-229) and the Vici Innovation Program (grant 016-130-669 to BF). Additional support was received from the European Community's Seventh Framework Programme (FP7/2007 – 2013) under grant agreements n° 602805 (Aggressotype), n° 602450 (IMAGEMEND), and n° 278948 (TACTICS) as well as from the European Community's Horizon 2020 Programme (H2020/2014 – 2020) under grant agreements n° 643051 (MiND) and n° 667302 (CoCA). The work was also supported by the Lundbeck Foundation (grant R102-A9118 and R155-2014-1724 to iPSYCH/A.D.B.), by a grant to the ENIGMA Consortium (grant number U54 EB020403) from the BD2K Initiative, a cross-NIH partnership, and by the ECNP Network 'ADHD across the Lifespan'. SEM is supported by an Australian National Health and Medical Research Council fellowship (SRFB-1103623). This work was partly carried out on the Dutch national e-infrastructure with the support of SURF Foundation.

The Enhancing NeuroImaging Genetics through Meta-Analysis (ENIGMA) Consortium provided summary statistics of the consortium findings to this project. The original publication of those findings as well as the list of contributing samples and authors may

be found on the ENIGMA website: http://enigma.ini.usc.edu. The Neurology Working Group of the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium also contributed with an independent set of summary statistics of consortium findings. The contributing cohorts and authors may be found on previous publications of the CHARGE consortium as listed on the website: http://www.chargeconsortium.com/. The ADHD working group of the Psychiatric Genomics Consortium (PGC) and the iPSYCH-SSI-Broad collaboration ADHD Working Group contributed with an independent set of summary of consortium findings. The data and a complete list of contributing samples and people can be obtained from the PGC download website (https://www.med.unc.edu/pgc/results-and-downloads).

Author contributions

Study conception and supervision: M.K., A.A.-V., B.F. Obtained funding: B.F., A.D.B. Provided samples and/or data: D.D, D.P.H., H.H.A., M.M., S.E.M. Conducted analyses: M.K. Contributed to analyses and data interpretation: R.K.W., J.L.S. Writing group: M.K., S.V.F., A.A.-V., B.F., A.D.B., J.B., N.R.M., R.S., E.S.-B., P.M.T. All authors reviewed and approved the final version of this manuscript.

Conflict of interest

Barbara Franke discloses having received educational speaking fees from Merz and Shire. Derrek P Hibar is now an employee of Janssen Research and Development, LLC. None of the other authors report conflicts of interest.

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Supplementary material

Participant samples

ADHD Working Group of the PGC and the ADHD iPSYCH-SSI-Broad collaboration

ADHD GWAS-MA summary statistics data were acquired from the ADHD Working Group of the PGC and the ADHD iPSYCH-SSI-Broad collaboration (n=55,374 (Demontis et al., 2017), https://www.med.unc.edu/pgc/results-and-downloads). Detailed quality control and imputation parameters are described in the original publication (Demontis et al., 2017). Briefly, genotype imputation was done using the bioinformatic pipeline "ricopili" and with the pre-phasing/imputation stepwise approach implemented in IMPUTE2/SHAPEIT using the haplotypes from the 1000 Genomes Project, phase 3, version 5 (1KGP3v5) (The 1000 Genomes Project Consortium, 2015) data. Association analyses using the imputed marker dosages were performed separately for the 11 PGC samples and the 23 waves in iPSYCH by an additive logistic regression model using PLINK v1.9 (Chang et al., 2015), with the derived principal components included as covariates as described in the original publication (Demontis et al., 2017). Subsequently, meta-analysis, including summary statistics from GWASs of the 23 waves in iPSYCH and 11 PGC samples, was conducted using an inverse-weighted fixed effects model. In total, 20,183 cases and 35,191 controls were used for the original analysis (**Supplementary Table 1**). Only SNPs with imputation quality (INFO score) >0.8 and MAF >0.01 were included in the meta-analysis. PGC+iPSYCH ADHD GWAS-MA summary statistics data only included markers which were supported by an effective sample size greater than 70% (8,047,420 markers) (Demontis et al., 2017).

ENIGMA

GWAS-MA summary statistics data on ICV and volumes of nucleus accumbens, amygdala, caudate nucleus, hippocampus, and putamen were from ENIGMA (http://enigma.ini.usc.edu/) (Hibar et al., 2015). In the GWAS-MAs on subcortical volumes those volumes had been adjusted for ICV to identify specific genetic contributions to individual volumes. The five subcortical volumes indicated and ICV were selected for the current study based on a recent mega-analysis reporting significant volume reductions in patients with ADHD compared to healthy controls (Hoogman et al., 2017). Access to the summary statistics of ENIGMA can be requested via their website (http://enigma.ini.usc.edu/download-enigma-gwas-results/). For the initial GWAS-MA analysis, MRI brain scans and genome-wide genotype data were available for 11,840 subjects from 22 cohorts. Genomic data were imputed to a reference panel (1000 Genomes, phase1, v3 (1KGP1v3) (Genomes Project Consortium et al., 2010)) comprising only European samples and with monomorphic SNPs removed. Imputation was performed at each site using MaCH for phasing and minimac for imputation (Fuchsberger et al., 2015). Only SNPs with an imputation score of RSQ >0.5 and minor allele counts >10 within each site were included. Tests of association were conducted separately for eight MRI volumetric phenotypes (nucleus accumbens, amygdala, caudate nucleus, hippocampus, pallidum, putamen, thalamus and ICV) with the following covariates in a multiple linear regression framework: age, age2, sex, four MDS components (to account for population structure) and ICV (for subcortical brain phenotypes). GWA statistics from each of the 22 sites were combined using a fixed-effect inverse variance-weighted meta-analysis as implemented in METAL (Willer et al., 2010). Prior to all analyses, a cohort including ADHD cases (NeuroIMAGE cohort, n=154) was removed from the ENIGMA data.

CHARGE

We obtained genome-wide GWAS-MA summary statistics data on ICV and hippocampal volume from the CHARGE Consortium (n=12,803 and n=13,039, respectively (Adams et al., 2016; Hibar et al., 2017)) and CHARGE summary statistics data had been requested by the principal investigator of the study described by Adams et al. (Adams et al., 2016). Genotyping was performed using a variety of arrays across contributing sites. Samples and variants underwent quality control procedures based on genetic homogeneity, call rate (< 95%), MAF <0.01, and Hardy-Weinberg Equilibrium (HWE p-value <1×10−6). Good quality variants were used as input for imputation to the 1000 Genomes reference panel (1KGP1v3; (Genomes Project Consortium et al., 2010)) using different software packages (MaCH/minimac, IMPUTE2, BEAGLE, GenABLE). Only SNPs with an imputation score of RSQ >0.5 and MAF>0.5% within each site were included in the meta-analysis. Full details on the site-specific genotyping and quality control can be found in Supplementary Table 2 of the original publication (Adams et al., 2016). GWAS of ICV and hippocampal volumes were performed for each site separately, controlling for age, sex, and, when applicable age^2, population stratification variables, study site, and diagnosis (when applicable). Summary statistics, including effect estimates of the genetic variant with ICV or hippocampal volume under an additive model, were exchanged to perform a fixed-effects meta-analysis weighting for sample size in METAL (Willer et al., 2010). After the final meta-analysis, variants were excluded if they were only available for fewer than 5,000 individuals.

Removal of duplicated individuals

Subject overlap between all PGC ADHD and ENIGMA cohorts was evaluated using a checksum algorithm to ensure the robustness of our results, given that some analyses were sensitive to the presence of duplicate individuals. For each individual, ten checksum numbers were created based on ten batches of 50 SNP genotypes and compared between individuals from both consortia. Based on these comparisons no subjects needed to be removed from the data sets. As no Danish cohort was included in the ENIGMA or CHARGE study, we assumed that there is no sample overlap between cohorts studying brain volume and iPSYCH.

GWAS meta-analysis of ENIGMA and CHARGE data sets

To increase the sample size for the hippocampal volume and ICV data, summary statistics of GWAS-MA results from ENIGMA (Hibar et al., 2015) (after removal of ADHD cases) and CHARGE (Adams et al., 2016; Hibar et al., 2017) were combined using a fixed-effects sample size-weighted meta-analysis framework as implemented in METAL (Willer et al., 2010). After the final meta-analysis, variants were excluded if they were only available for fewer than 5,000 individuals or a MAF ≤0.005. After filtering, the meta-analyses results included more than 9,145,464 markers. Importantly, the ENIGMA and CHARGE discovery data sets only included cohorts of European ancestry (all individuals had both imaging and genetics data). This overview is presented in the original publication of Adams and colleagues (Adams et al., 2016) in Supplementary Table 1.

Linkage disequilibrium score regression (LDSR)

For LDSR, each GWAS-MA data set underwent additional filtering. Only markers overlapping with HapMap Project Phase 3 SNPs and passing the INFO score ≥0.9 and MAF ≥0.01 filters were included (where available). SNPs with missing values, duplicate rs-numbers, too low a sample size (where available SNPs with an effective sample size less than 0.67 times the 90th percentile of sample size were removed), or that were strand-ambiguous - as well as indels - were removed. As described in the original ADHD GWAS-MA paper (Demontis et al., 2017), for LDSR analysis the European only subset was used (n_{cases}=19,099 and $n_{controls}$=34,194), since LDSR requires linkage disequilibrium [LD] data from a sample of comparable ethnic background). For the ENIGMA amygdala results, the mean χ^2 was too low (1.0) to reliably estimate SNP heritability using LDSR. **Table 1** shows genetic correlations between the regional brain volumes; subcortical volumes are not strongly genetically correlated with ICV. The analyses used a two-step procedure with the LD-scoring analysis package (Bulik-Sullivan et al., 2015). An unconstrained regression estimated regression intercepts for each pair of phenotypes. Since we adopted protocols to exclude sample overlap, we also performed the analyses with regression intercept for the genetic correlation analysis defined as zero (**Supplementary Table 2**). To compute p-values, standard errors were estimated using a block jackknife procedure.

SNP effect concordance analysis (SECA)

Post-processing of genetic data

To statistically compare the ADHD and six brain volume GWAS-MAs, we used SNPs passing quality control and filtering rules (for ADHD GWAS-MA INFO ≥0.9 and MAF ≥0.01 and for ENIGMA and CHARGE GWAS-MA RSQ ≥0.5 and MAF ≥0.005) in all data sets. With these data, we performed a clumping procedure in PLINK (Purcell et al., 2007) to identify an independent SNP from every LD block across the genome. The clumping procedure was performed separately for each of the brain volume GWAS-MAs using a 500 kb window,

with SNPs in LD (r^2 >0.2) in the European reference samples from the 1KGP1v3 (Genomes Project Consortium et al., 2010). The SNP with the lowest p-value within each LD block was selected as the index SNP representing that LD block and all other SNPs in the LD block were dropped from the analysis. The result after applying the clumping procedure was sets of independent SNPs representing the total variation explained across the genome conditioned on the significance in each brain volume GWAS-MA. For each of these sets of SNPs, we then determined the corresponding ADHD GWAS-MA test statistic for each independent, index SNP and used these data sets for the subsequent analyses.

Tests of pleiotropy and concordance

We used SNP Effect Concordance Analysis (SECA) (Nyholt, 2014) to determine the extent and directionality of genetic overlap between ADHD and each brain volume. Within SECA we performed a global test of pleiotropy using a binomial test at 12 p-value levels: P ≤(0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9). For a given brain volume and ADHD paired set, we separately ordered SNPs based on their p-value for association with each trait. For each of the 12 p-value levels, we determined the total number of SNPs overlapping between the two traits at each p-value threshold and compared that number to the expected random overlap under the null hypothesis of no pleiotropy using a binomial test. In total, 144 comparisons were performed. We tallied the number of comparisons with evidence of overlap at a nominally significant level of P ≤0.05. To evaluate the global level of pleiotropy, we generated 1,000 permuted data sets for a given brain volume to ADHD comparison and determined, if the number of significance thresholds with genetic overlap was significantly greater than chance.

Similarly, we estimated concordance, the agreement in SNP effect directions across two traits. We determined whether or not there was a significant (P ≤0.05) positive or negative trend in the effect of the overlapping SNPs at each of the 12 p-value thresholds. This was done using a two-sided Fisher's exact test. The direction of effect for each SNP was determined by the sign of the SNP regression coefficient (OR or beta value) from each meta-analysis. In the ADHD GWAS-MA, an odds ratio >1 for a SNP indicates that the A1 reference allele was associated with an increased risk of developing ADHD (an odds ratio <1 indicates a protective allele). A positive Beta value for a SNP in a brain volume GWAS-MA indicates that the A1 reference allele of that SNP is associated with an increase in brain volume (a negative Beta value indicates that the A1 reference allele of that SNP is associated with a reduction in brain volume). We estimated the global level of concordance between a given brain trait and ADHD by generating 1 000 permuted data sets, repeating the Fisher's exact test procedure, and determined if the number of significant overlapping thresholds was significantly greater than chance (see Nyholt et al., 2014 (Nyholt, 2014) for details of the SECA analysis).

In total, we tested for pleiotropy and concordance between ADHD and six brain volumes. In accordance with the number of tests performed, we set a Bonferroni-corrected significance level at $P=0.05/(2*6)=4.17x10^{-3}$.

Independent genome-wide significant markers and loci

LD-independent markers associated at $P <1x10^{-5}$ were defined using the clump flag in PLINK v1.9 (Chang et al., 2015). Clumping was used to group additional associated markers within a 0.5 Mb window surrounding the index SNP. Markers were grouped to the index SNPs if they were also associated ($P <0.001$) and were in LD with the index SNP ($r^2 >0.1$). A genome-wide significant locus was defined as the physical region containing the identified LD independent index SNPs and their correlated variants ($r^2>0.8$) with $P <0.001$. Associated loci within 250 kb of each other were merged. All LD statistics were calculated using the 1KGP3v5 (The 1000 Genomes Project Consortium, 2015) reference haplotypes.

SNP sign test in the intelligence GWAS-MA

We performed sign tests to investigate a potential accumulation of same or opposite direction effects of SNPs between ADHD+ICV, ADHD, and ICV GWAS-MA data and the intelligence GWAS-MA data (Savage et al., 2018). The ADHD+ICV GWAS-MA data were clumped to define independent loci (Supplementary Methods) for all variants with $P<1x10^{-5}$ and $P<1x10^{-4}$ in the ADHD+ICV GWAS-MA using 1KGP3v5 data on European ancestry populations as reference. Based on the negative genetic correlation between ADHD and intelligence, we expected an overrepresentation of discordant SNP effects. In contrast, the positive genetic correlation between ICV and intelligence guided us in specifically looking for concordant SNP effects. However, for the ADHD+ICV GWAS-MA data set we did not favor any directionality a priori and therefore tested for both same and opposite direction effects in the intelligence data set (Savage et al., 2018). The proportion of variants with a concordant or discordant direction of effect in the intelligence GWAS-MA was evaluated using a binomial test against a null hypothesis of 0.5 (i.e. chance level). This test was done for SNPs, which (1) passed the p-value threshold of $P<1x10^{-5}$ (64 LD-independent SNPs) and $P<1x10^{-4}$ (327 LD-independent SNPs) in the ADHD+ICV GWAS-MA and (2) showed smaller p-values in the ADHD+ICV GWAS-MA compared to the ADHD and ICV GWAS-MA individually (43 and 225 LD-independent SNPs). We set a Bonferroni-corrected significance level at $P=0.05/(2*3)=0.00833$.

Weighted meta-analysis of ADHD and brain volume data sets

Independent of the results of the global overlap analyses, we also performed meta-analyses combining the results from the ADHD GWAS-MA with results from GWAS-MAs of brain volumes (amygdala, nucleus accumbens, caudate nucleus, hippocampus, putamen, and ICV). This was done using a modified sample size-based weighting method, integrating the

binary ADHD trait (ADHD risk) with the continuous trait (brain volume traits), as described in Demontis et al.(Demontis et al., 2017). For the meta-analyses, modified sample size-based weights were derived to account for the respective heritability, genetic correlation, and measurement scale of the GWASs. The adjusted samples sizes reflect differences in power between the studies due to measurement scale and relative heritability that is not captured by sample size. Thereby, the contribution of the continuous phenotype's GWAS to the meta-analysis is reduced based on imperfect correlation with the dichotomous phenotype of interest (in this case ADHD risk). The adjustments are computed based on the sample and population prevalence of the dichotomous phenotype, the estimated SNP heritability of the two phenotypes (liability scale for dichotomous phenotype), and the genetic correlation between the two phenotypes, as well as the average SNP LD score, and the number of SNPs. Heritability and genetic correlation values to compute these weights are computed using LD score regression (Bulik-Sullivan et al., 2015) as described before. For a comprehensive description of the method for meta-analysis of continuous and dichotomous phenotype and notes on the implementation please see the supplementary information of the original ADHD GWAS-MA publication (Demontis et al., 2017). For all brain volumes, we also performed naive meta-analyses given the low genetic correlations with ADHD risk observed. Correcting for meta-analyzing six brain phenotypes with ADHD, we set the threshold for genome-wide significance at $P=5x10^{-8}/6=8.33x10^{-9}$. Additionally, loci were considered cross-disorder relevant if (i) those loci were genome-wide significant in the cross-phenotype meta-analysis, (ii) and/or had a cross-phenotype p-value, which was improved by at least one order of magnitude, and (iii) had a cross-phenotype z-score, which (at least) equaled the ones observed in the GWAS-MAs for the individual phenotypes. The LD score intercept and ratio of the individual and meta-analyzed summary statistics are presented in **Supplementary Table 23** in order to compare the estimates of the overall genetic signal. The percentage variance explained by each genome-wide significant index SNP was determined based on the ENIGMA2 data set after correction for covariates using the following equation:

$$\frac{R^2_{g|c}}{1 - R^2_c} = (t^2/((n - k - 1) + t^2)) * 100$$

where the *t*-statistic is calculated as the beta coefficient for a given SNP from the regression model (controlling for covariates) divided by the standard error of the beta estimate, and where *n* is the total number of subjects and *k* is the total number of covariates included in the model (k=10). $R^2_{g|c}$ is the variance explained by the variant controlling for covariates, and R^2_c is the variance explained by the covariates alone. $R^2_{g|c}/(1 - R^2_c)$ gives the variance explained by the genetic variant after accounting for covariate effects.

Gene-based and gene-set analyses for ADHD+brain GWAS-MA data

Genome-wide summary statistics of (i) ADHD GWAS-MA, (ii) individual brain GWAS-MAs, and (iii) weighted meta-analysis data for combined ADHD and brain volume GWAS-MAs were used as input for gene-based analyses. For the ADHD+brain GWAS-MA, only SNPs shared between ADHD and brain volume data sets were included. Statistical analyses were performed using the Multi-marker Analysis of GenoMic Annotation (MAGMA) software package (version 1.05, (de Leeuw et al., 2015)). Genome-wide SNP data from a reference panel 1KGP1v3 (Genomes Project Consortium et al., 2010) was annotated to NCBI Build 37.3 gene locations using a symmetric 100 kb flanking window. Both files were downloaded from http://ctglab.nl/software/magma. The gene annotation file was used to map genome-wide SNP data from the different studies (ADHD GWAS-MA, brain GWAS-MAs, and ADHD+brain GWAS-MA), to assign SNPs to genes followed by the calculation of gene-based p-values. This step was done for each of the data sets individually. For the gene-based analyses, single SNP p-values within a gene were transformed into a gene-statistic by taking the mean of the χ^2-statistic among the SNPs in each gene. To account for LD, the 1KGP1v3 (Genomes Project Consortium et al., 2010) was used as a reference to estimate the LD between SNPs within (the vicinity of) the genes (http://ctglab.nl/software/MAGMA/ref_data/g1000_ceu.zip). Gene-wide p-values were converted to z-values reflecting the strength of the association of each gene with the phenotype, with higher z-values corresponding to stronger associations. Genome-wide gene-based results were considered significant if they reached the Bonferroni-corrected P-value-threshold for testing 18,310 genes ($P<2.731x10^{-6}$; for gene-based results of all genes see **Supplementary Table 4-9**). Then, we assessed the number of significant genes overlapping between the ADHD GWAS-MA results and the cross-trait ADHD+brain GWAS-MA results. Of those overlapping genes, we considered those as cross-trait relevant if (i) those genes were genome-wide significant in the cross-trait MA, (ii) and had a cross-trait association p-value that was smaller compared to the separate analyses of ADHD and brain volume, and (iii) had a nominally significant ($P<0.05$) P-value in the individual gene-based brain trait result. The latter criterion was established in order to distinguish the 'true' cross-trait effect from increase in association signal that is purely related to an increase in samples size when combining the two GWAS-MA data sets. Genes, meeting these criteria, were reported and selected for further investigation.

Based on our finding that *SEMA6D* is a key locus contributing to both ADHD risk and ICV – a loci involved in neuronal migration and axonal path finding – we investigated, whether neurite outgrowth-related genes in general have a role in ADHD–ICV genetic overlap. For the gene-set analyses we chose to use a pre-defined gene-set of 45 neurite outgrowth genes . In the initial study, Poelmans and colleagues investigated the presence of genomic convergence in the top findings of the five published GWASs of ADHD. Then, they carried out bioinformatics pathway analyses, using the Ingenuity and BiNGO tools, as well as a systematic literature analysis of 85 genes from the five published GWASs containing SNPs associated with ADHD at a p value <0.0001. Out of those 85 top-ranked ADHD candidate genes, 45 genes encode proteins that fit into a neurodevelopmental network involved in directed neurite outgrowth. Moreover, the authors added data on CNVs in patients with ADHD and data from animal studies and this provided further support for the involvement of this network in ADHD etiology. Additionally, they could show that several network proteins are directly modulated by stimulants (commonly used treatment for ADHD).

Subsequent to the genome-wide gene-based analysis, we also tested, whether genes in the neurite-outgrowth gene-set (defined previously, $N_{genes}=45$ (Poelmans et al., 2011)) were jointly associated with results of the weighted meta-analytic data of ADHD+ICV using self-contained and competitive testing (de Leeuw et al., 2016). For the gene-set analyses, we used an intercept-only linear regression model including a subvector corresponding to the genes in the gene-set. This self-contained analysis tests whether the gene-set shows any association with the phenotype at all by evaluating whether the regression coefficient of this regression is larger than 0. Next, we tested whether genes in the gene-set were more strongly associated with phenotype than all other genes in the genome. Therefore, the regression model was then expanded including all genes outside the gene-set. With this competitive test, the differences between the association of the neurite-outgrowth gene-set to genes outside this gene-set is tested, accounting for the polygenic nature of such a complex trait. To account for the potentially confounding factors of gene size and gene density, both variables as well as their logarithms were included as covariates in the competitive gene-set analysis. Since self-contained tests do not take into account the overall level of association across the genome, gene size (number of principal components, or SNPs), and gene density, we were particularly interested in the competitive test for the current analysis. Basically, a significance in the self-contained tests but not the competitive test, suggests that the effect of the gene-set is not different from the background effect that captures the polygenic nature of ADHD. Moreover, a non-significant competitive p-value can be interpreted as not being able to disentangle the part of the polygenicity attributable to the genes in the gene set versus the polygenicity "remaining" (i.e. not captured by the set) on the rest of the genome and not that the selected gene-set has no effect on the outcome. Subsequently, we tested whether the gene-set was associated with the two individual data sets as well. In this, the same procedure was followed for analysis of the ADHD GWAS-MA and ENIGMA+CHARGE ICV GWAS-MA summary statistics individually. *Post-hoc*, the individual genes in the set were investigated, by reviewing gene test-statistics of the weighted ADHD+ICV GWAS-MA results. Genes of the neurite-outgrowth set were considered gene-wide significant, if they reached the adjusted Bonferroni correction threshold ($P=0.05/45=0.00111$). Subsequently, we reviewed gene-based associations in the ADHD GWAS-MA and ENIGMA+CHARGE ICV GWAS-MA results separately. For genome-wide gene-based comparisons we considered results significant, if they reached the Bonferroni-corrected P-value-threshold for testing 18,411 genes ($P<2.716x10^{-6}$). Then, we assessed the

number of significant genes overlapping between the ADHD GWAS-MA results and the cross-trait ADHD+brain GWAS-MA results. Of those overlapping genes, we considered those as cross-trait relevant if (i) those genes were genome-wide significant in the cross-trait MA, (ii) and had a cross-trait association p-value that was smaller compared to the separate analyses of ADHD and brain volume, and (iii) had a nominally significant (P<0.05) P-value in the individual gene-based brain trait result. The latter criterion was established in order to distinguish the 'true' cross-trait effects from increase in association signal that is purely related to an increase in samples size when combining the two GWAS-MA data sets. Genes, meeting these criteria, were reported and selected for further investigation.

Significant genes with lower association p-values in the meta-analysis, compared to the separate analyses of ADHD and ICV, were reported in **Supplementary Table 10**.

Expression quantitative trait loci and brain gene expression

To assess potential functionality in (brain) tissues, we tested the identified risk variants (**Table 4**) for association with gene expression. Expression quantitative trait loci (eQTL) were examined using data from the GTEx portal (https://www.gtexportal.org/home/) (GTEx Consortium, 2013). The data is presented in Supplementary Table 7 and is shown as normalized effect sizes (NES) and p-values. NES describes the slope of the linear regression of normalized expression data versus the three genotype categories using single-tissue eQTL analysis, representing eQTL effect size. The normalized expression values are based on quantile normalization within each tissue, followed by inverse quantile normalization for each gene across samples. The p-value results from a t-test that compares observed beta from single-tissue eQTL analysis to a null beta of 0. In addition, blood eQTL data were queried using the Blood eQTL Browser (http://genenetwork.nl/bloodeqtlbrowser/) (Westra et al., 2013).

We also investigated the spatio-temporal expression pattern in brain tissue for genes with significantly associated variants in the approaches described earlier (**Table 4**) using data from the Human Brain Transcriptome Project (http://hbatlas.org). We assessed messenger RNA (mRNA) expression trajectories in six regions of the developing and adult human brain. Spanning periods from embryonic development to late adulthood, this data set provides genome-wide exon-level transcriptome data generated using the Affymetrix GeneChip Human Exon 1.0 SS Arrays from over 1,340 tissue samples sampled from both hemispheres of *postmortem* human brains (n=57) (Kang et al., 2011). Gene expression over the lifespan from the spatio-temporal atlas was graphed using custom R scripts (Kang et al., 2011).

URLs

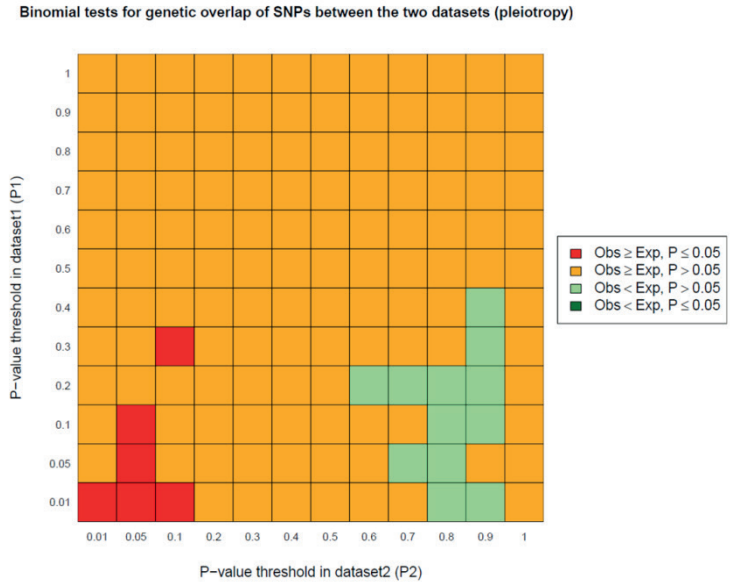
http://enigma.ini.usc.edu/download-enigma-gwas-results/
https://www.med.unc.edu/pgc/results-and-downloads
https://github.com/bulik/ldsc
https://neurogenetics.qimrberghofer.edu.au/SECA/
https://analysistools.nci.nih.gov/LDlink/
https://www.gtexportal.org/home/
http://hbatlas.org
http://ctglab.nl/software/MAGMA/ref_data/g1000_ceu.zip
http://locuszoom.sph.umich.edu/

Data availability

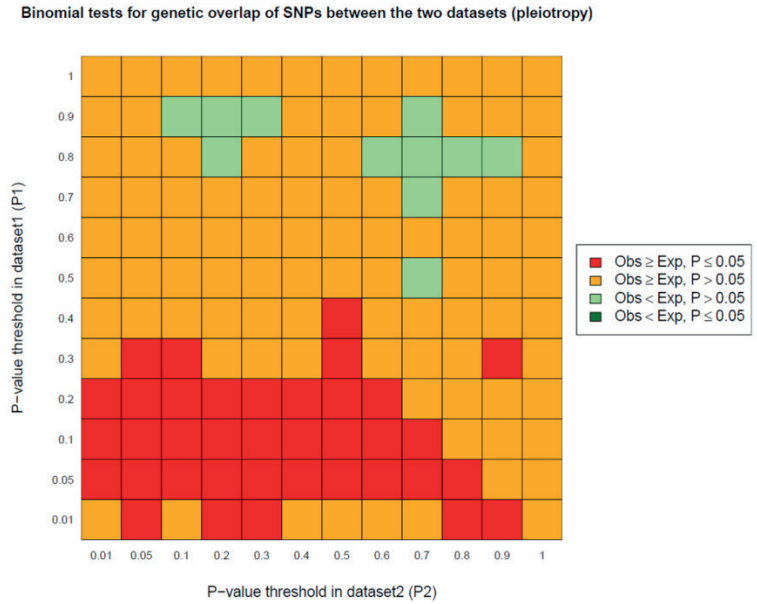
The genome-wide summary statistics that support the findings of this study are available at the consortia websites.
PGC ADHD working group and the ADHD iPSYCH-SSI-Broad collaboration: https://www.med.unc.edu/pgc/results-and-downloads
ENIGMA and ENIGMA+CHARGE for ICV and hippocampus: http://enigma.ini.usc.edu/download-enigma-gwas-results/

Supplementary figures

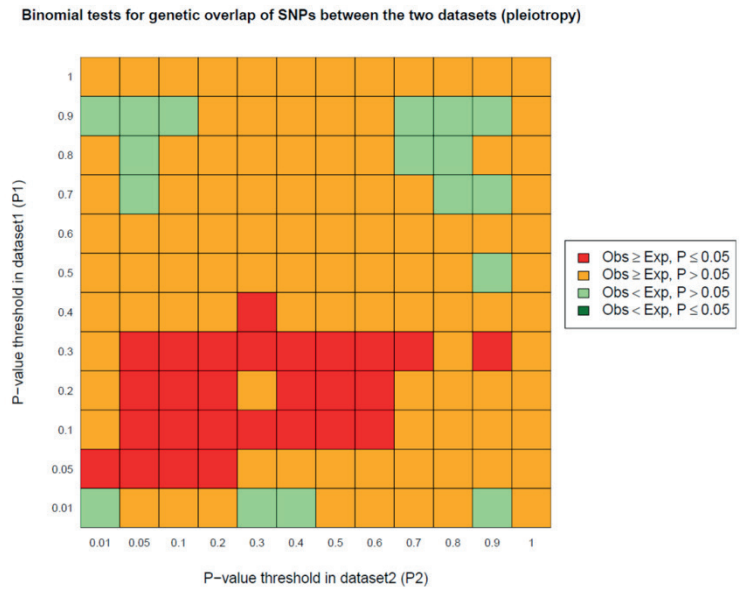
Supplementary Figure 1 (a-h): Global evidence of pleiotropy between the ADHD GWAS and each brain volume GWAS (nucleus accumbens, amygdala, caudate nucleus, hippocampus, putamen, and intracranial volume). Plots show the results from SECA separated into separate panels, one for each comparison.



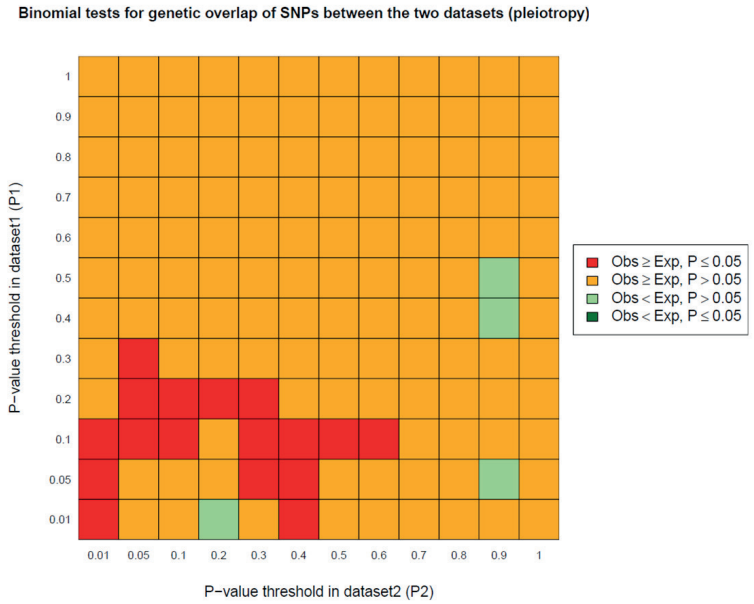
Supplementary Figure 1 (a): Global evidence of pleiotropy between ADHD GWAS and nucleus accumbens volume. P1 in the plot is the ADHD GWAS and P2 is the nucleus accumbens GWAS. The global evidence for pleiotropy was not significant after accounting for multiple testing (P=0.034).



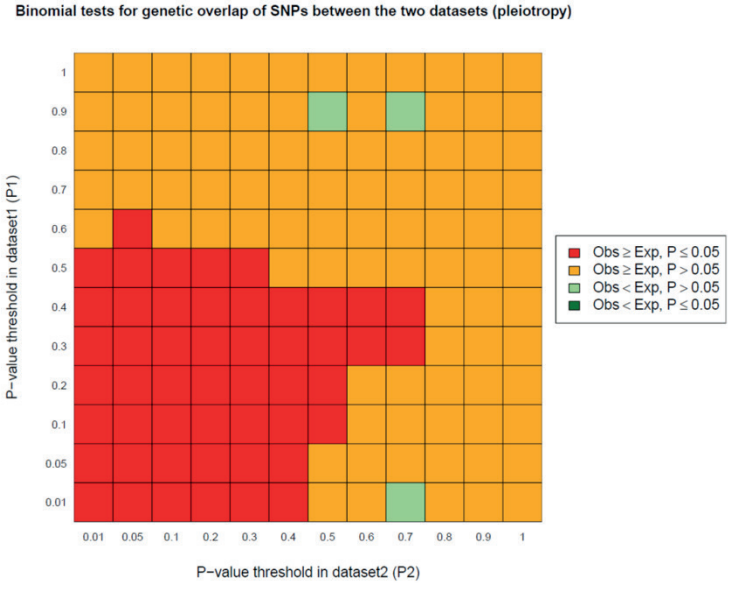
Supplementary Figure 1 (b): Global evidence of pleiotropy between ADHD GWAS and amygdala volume. P1 in the plot is the ADHD GWAS and P2 is the amygdala GWAS. The global evidence for pleiotropy was significant after accounting for multiple testing (P<0.001).



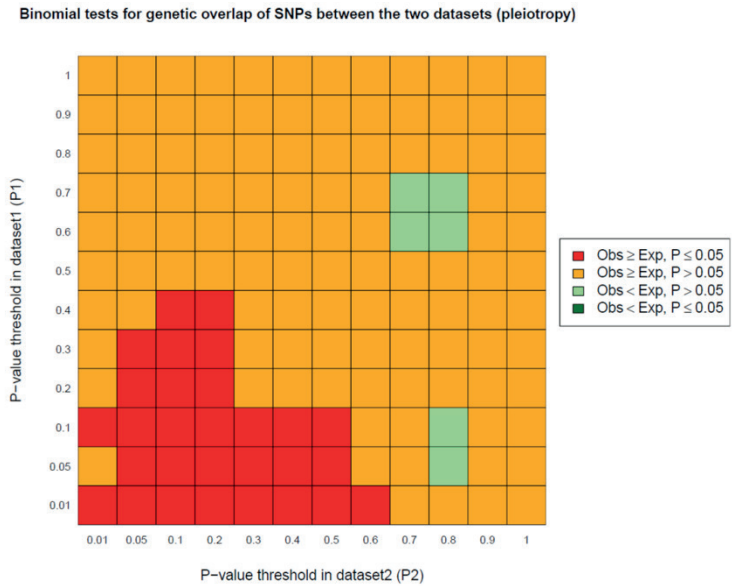
Supplementary Figure 1 (c): Global evidence of pleiotropy between ADHD GWAS and caudate nucleus volume. P1 in the plot is the ADHD GWAS and P2 is the caudate nucleus GWAS. The global evidence for pleiotropy was significant after accounting for multiple testing (P<0.001).



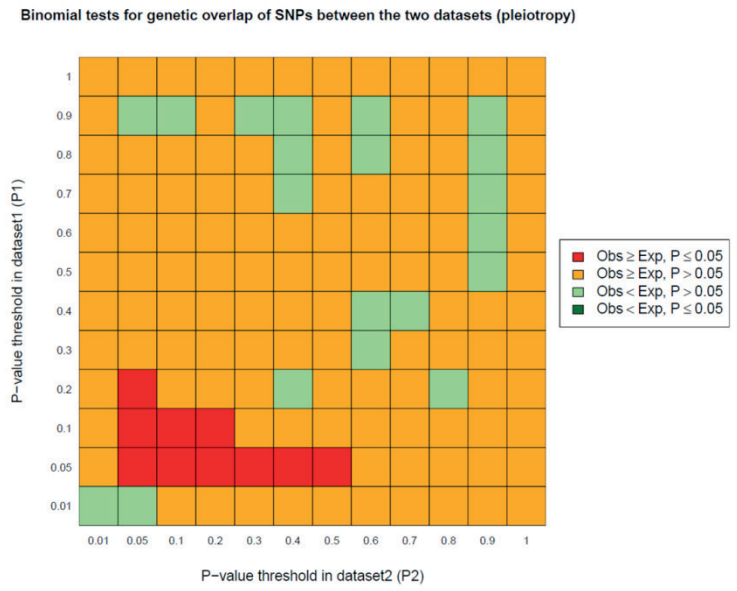
Supplementary Figure 1 (d): Global evidence of pleiotropy between ADHD GWAS and hippocampus volume (ENIGMA+CHARGE GWAS-MA). P1 in the plot is the ADHD GWAS and P2 is the hippocampus GWAS. The global evidence for pleiotropy was significant after accounting for multiple testing (P=0.002).



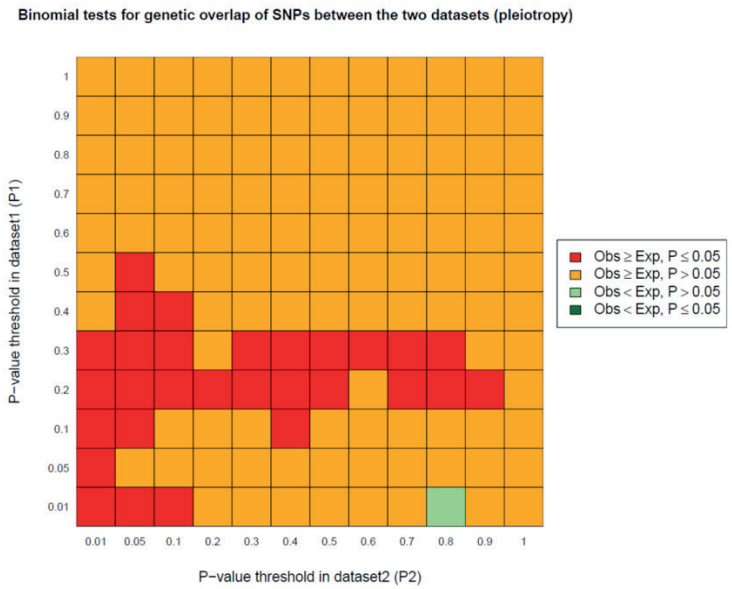
Supplementary Figure 1 (e): Global evidence of pleiotropy between ADHD GWAS and intracranial volume (ENIGMA+CHARGE GWAS-MA). P1 in the plot is the ADHD GWAS and P2 is the intracranial volume GWAS. The global evidence for pleiotropy was significant after accounting for multiple testing (P<0.001).



Supplementary Figure 1 (f): Global evidence of pleiotropy between ADHD GWAS and putamen volume. P1 in the plot is the ADHD GWAS and P2 is the putamen volume GWAS. The global evidence for pleiotropy was significant after accounting for multiple testing (P<0.001).

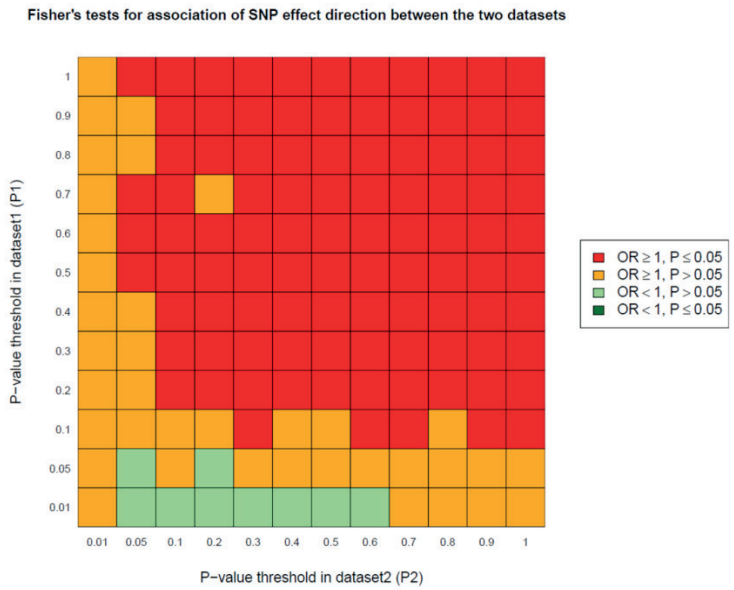


Supplementary Figure 1 (g): Global evidence of pleiotropy between ADHD GWAS and hippocampus volume (ENIGMA only GWAS-MA). P1 in the plot is the ADHD GWAS and P2 is the hippocampus GWAS. The global evidence for pleiotropy was not significant after accounting for multiple testing (P=0.005).

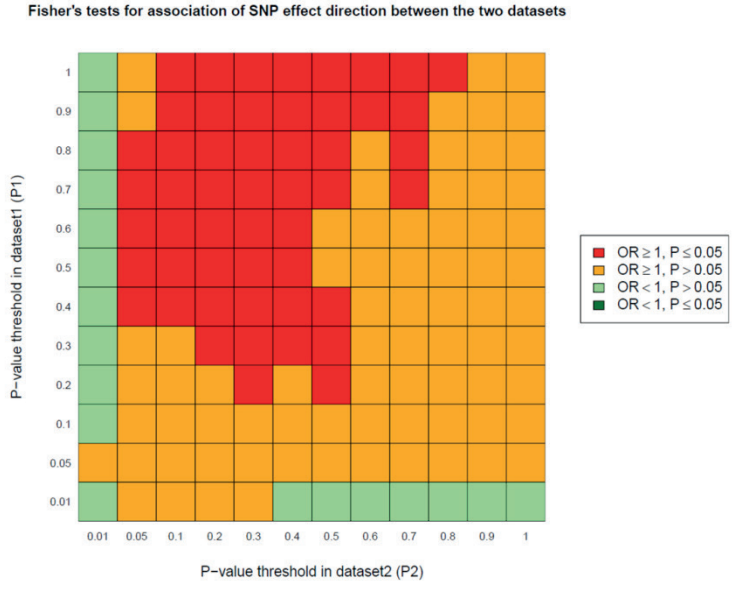


Supplementary Figure 1 (h): Global evidence of pleiotropy between ADHD GWAS and intracranial volume (ENIGMA only GWAS-MA). P1 in the plot is the ADHD GWAS and P2 is the intracranial volume GWAS. The global evidence for pleiotropy was significant after accounting for multiple testing (P<0.001).

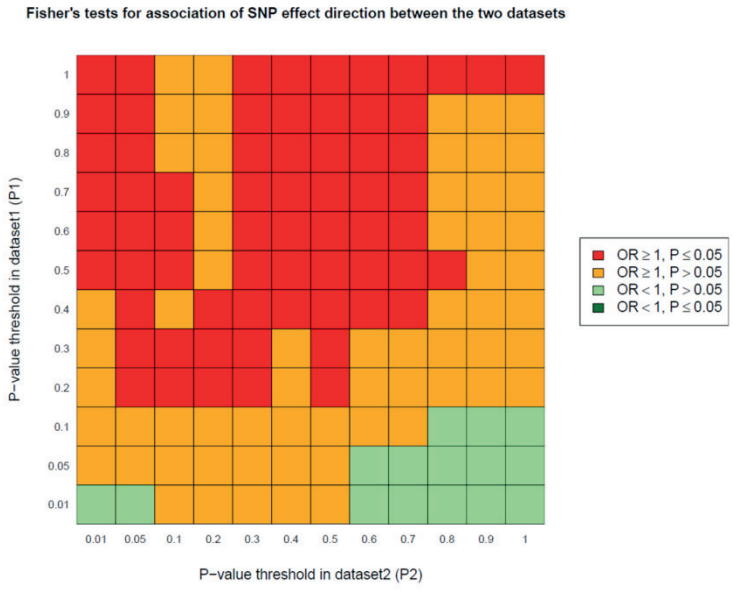
Supplementary Figure 2 (a-h): Global evidence of concordance between the ADHD GWAS and each brain volume GWAS (nucleus accumbens, amygdala, caudate nucleus, hippocampus, putamen, and intracranial volume). Plots show the results from SECA separated into separate panels, one for each comparison.



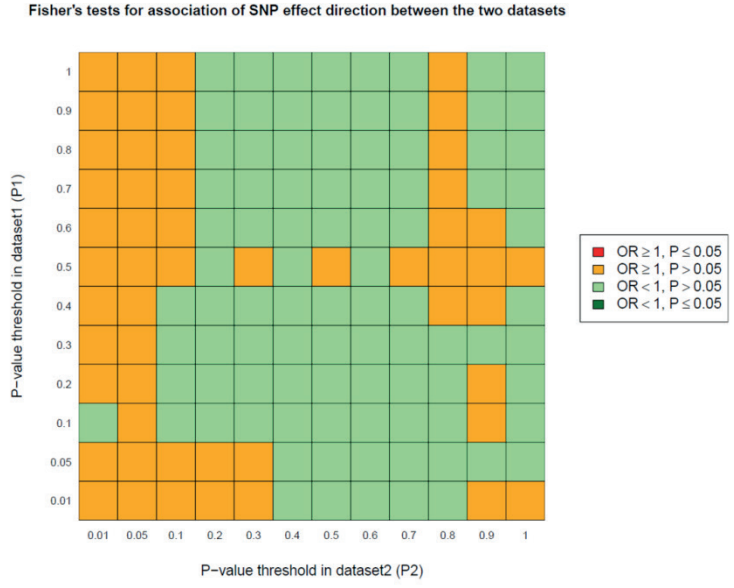
Supplementary Figure 2 (a): Global evidence for concordant effects between ADHD GWAS and nucleus accumbens. P1 in the plot is the ADHD GWAS and P2 is the nucleus accumbens GWAS. The global evidence for positive concordance was significant after accounting for multiple testing (P=0.002).



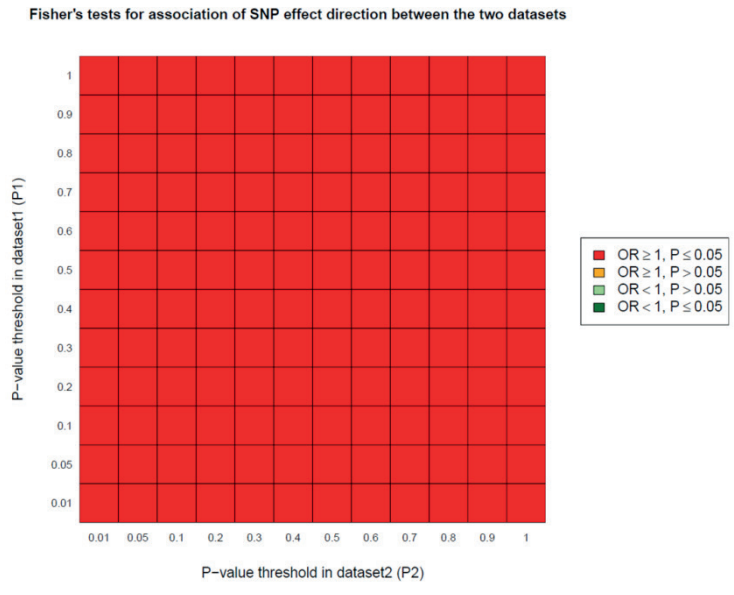
Supplementary Figure 2 (b): Global evidence for concordant effects between ADHD GWAS and amygdala. P1 in the plot is the ADHD GWAS and P2 is the amygdala GWAS. The global evidence for concordance was not significant after accounting for multiple testing (P=0.006).



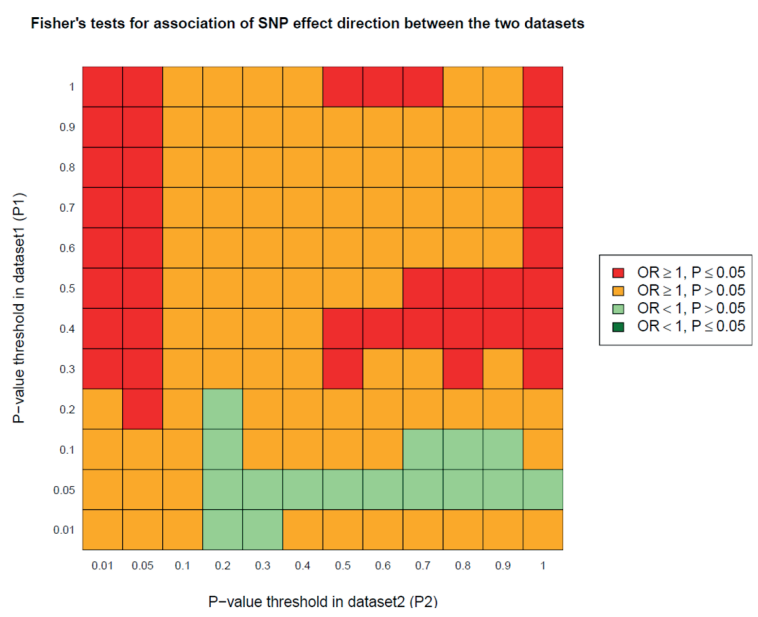
Supplementary Figure 2 (c): Global evidence for concordant effects between ADHD GWAS and caudate nucleus. P1 in the plot is the ADHD GWAS and P2 is the caudate nucleus GWAS. The global evidence for positive concordance was significant after accounting for multiple testing (P=0.004).



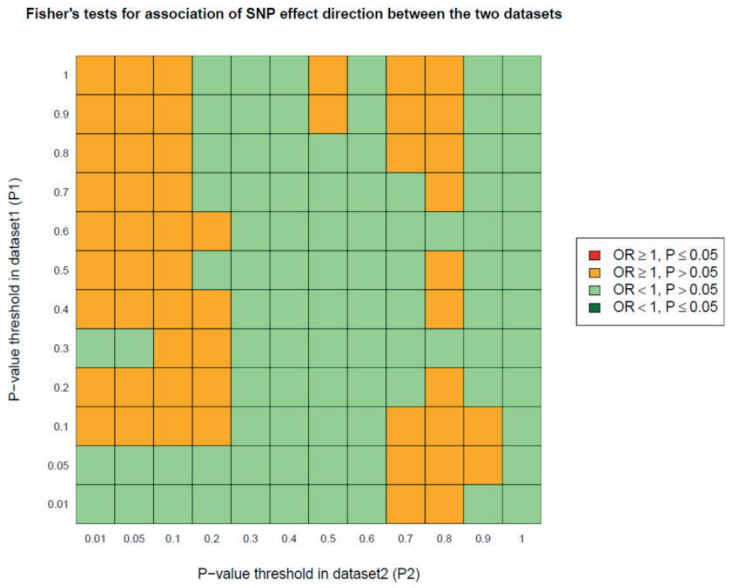
Supplementary Figure 2 (d): Global evidence for concordant effects between ADHD GWAS and hippocampus volume (ENIGMA+CHARGE GWAS-MA). P1 in the plot is the ADHD GWAS and P2 is the hippocampus volume GWAS. The global evidence for concordance was not significant after accounting for multiple testing (P=1).



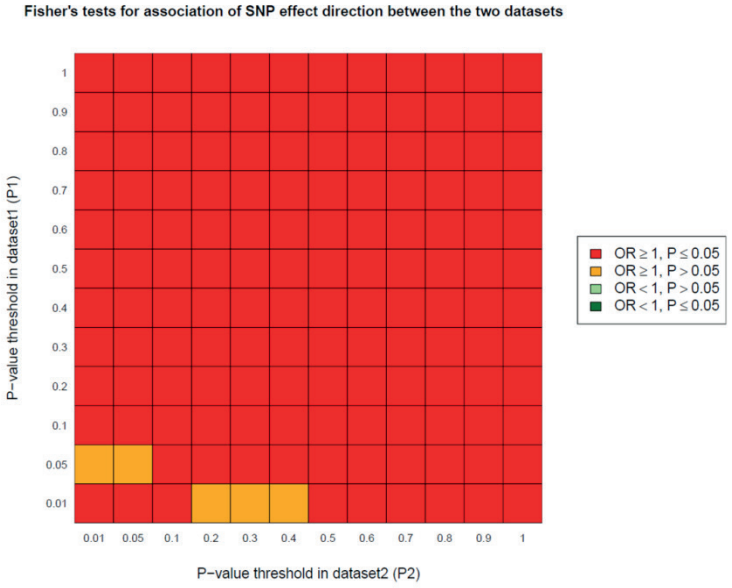
Supplementary Figure 2 (e): Global evidence for concordant effects between ADHD GWAS and intracranial volume (ENIGMA+CHARGE GWAS-MA). P1 in the plot is the ADHD GWAS and P2 is the intracranial volume GWAS. The global evidence for negative concordance was significant after accounting for multiple testing (P<0.001).



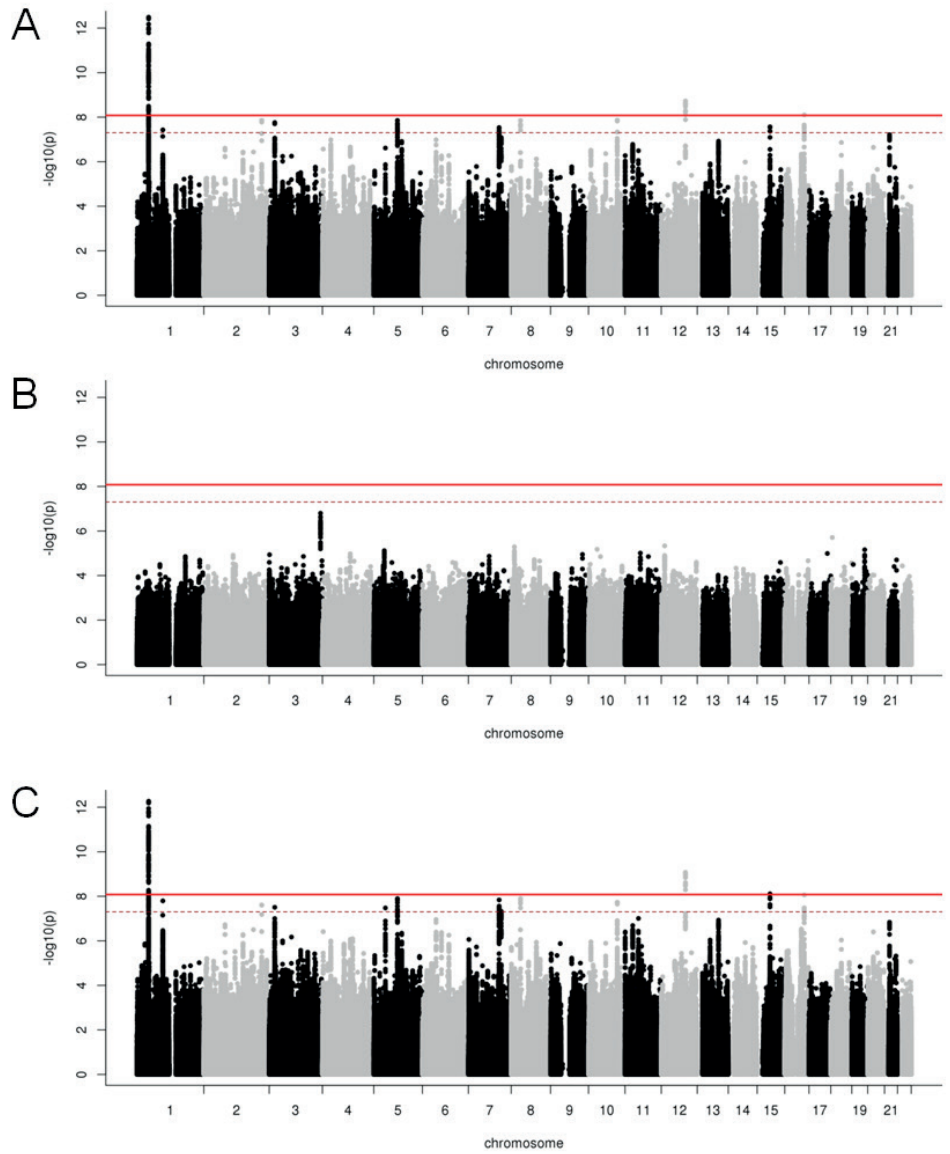
Supplementary Figure 2 (f): Global evidence for concordant effects between ADHD GWAS and putamen. P1 in the plot is the ADHD GWAS and P2 is the putamen GWAS. The global evidence for concordance was not significant after accounting for multiple testing (P<0.001).



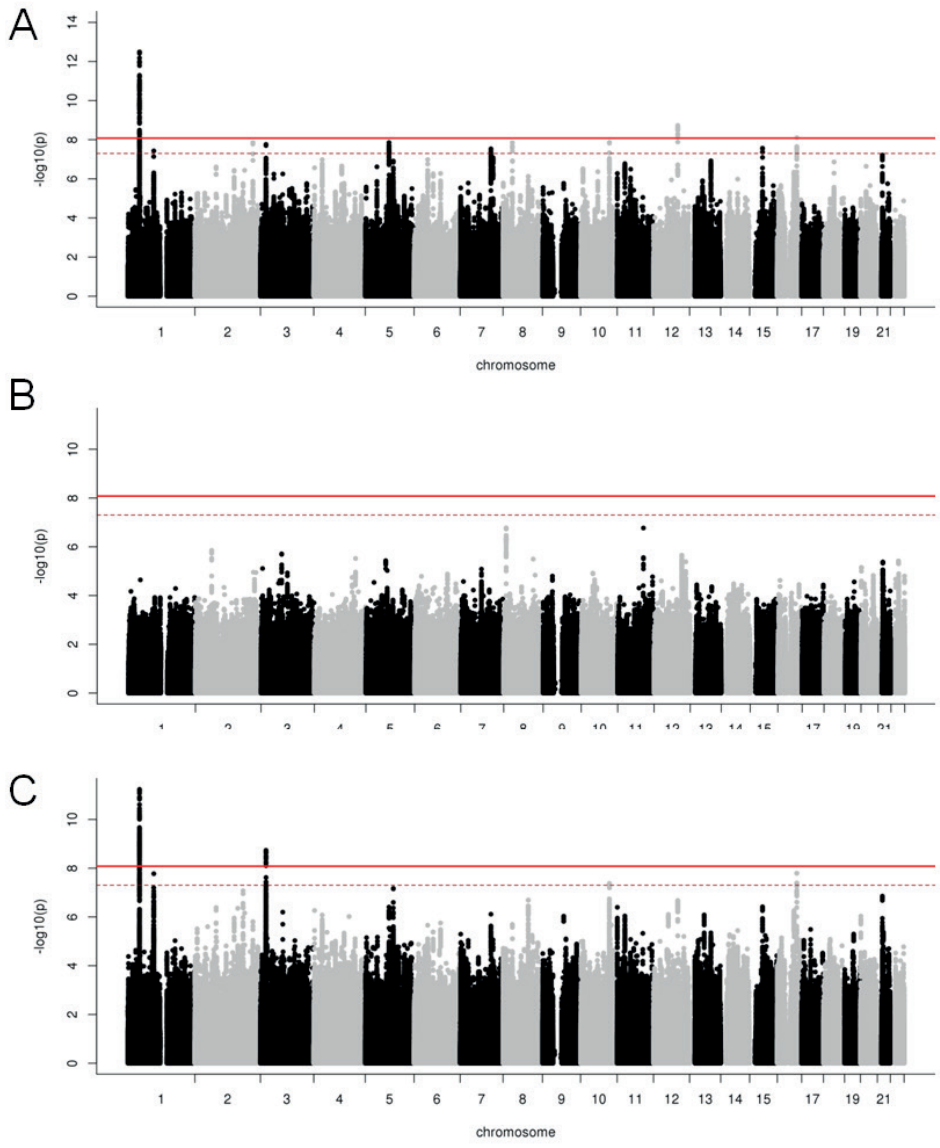
Supplementary Figure 2 (g): Global evidence for concordant effects between ADHD GWAS and hippocampus volume (ENIGMA only GWAS-MA). P1 in the plot is the ADHD GWAS and P2 is the hippocampus volume GWAS. The global evidence for concordance was not significant after accounting for multiple testing (P=1).



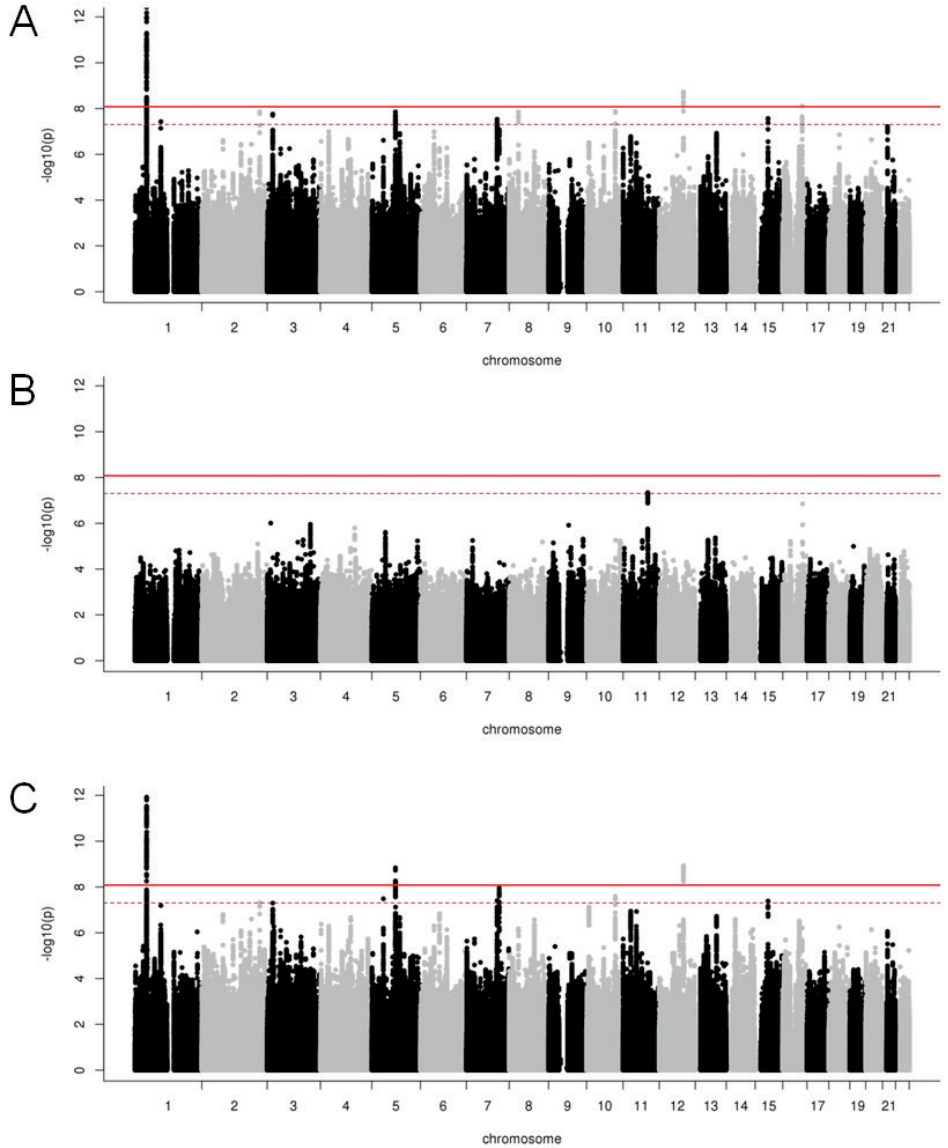
Supplementary Figure 2 (h): Global evidence for concordant effects between ADHD GWAS and intracranial volume (ENIGMA only GWAS-MA). P1 in the plot is the ADHD GWAS and P2 is the intracranial volume GWAS. The global evidence for negative concordance was significant after accounting for multiple testing (P<0.001).



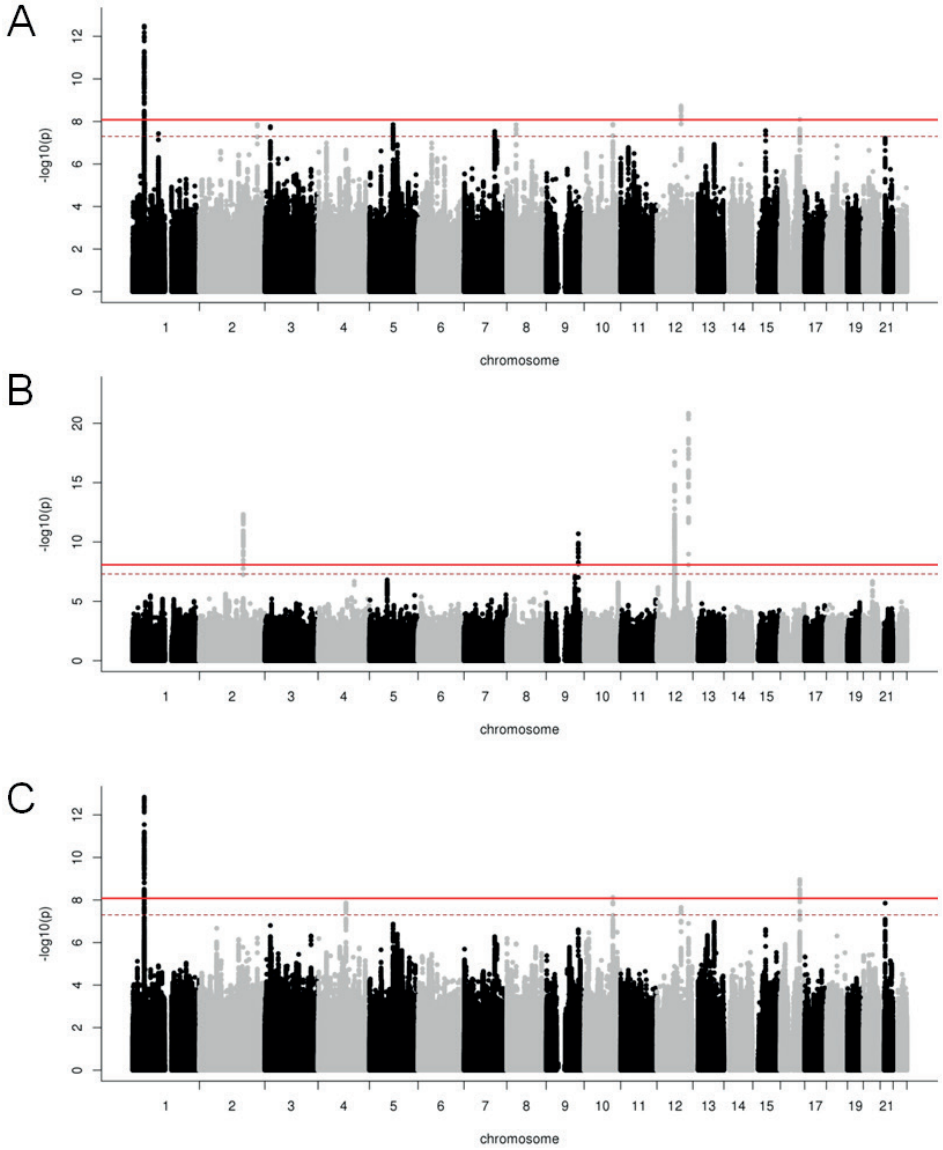
Supplementary Figure 3: Common genetic variants associated with ADHD, nucleus accumbens and the meta-analysis of ADHD and nucleus accumbens. Manhattan plots in which every point represents a single genetic variant plotted according to its genomics position (x-axis) and its $-\log_{10}$(P) for association with the respective trait (y-axis). The solid bright red line represents the study-wide genome-wide significance of $P<8.33\times10^{-9}$ and the dashed dark red line represents the genome-wide significance of $P<5\times10^{-8}$. (A) PGC+iPSYCH ADHD GWAS-MA. (B) ENIGMA nucleus accumbens GWAS-MA. (C) ADHD+ nucleus accumbens weighted GWAS-MA.



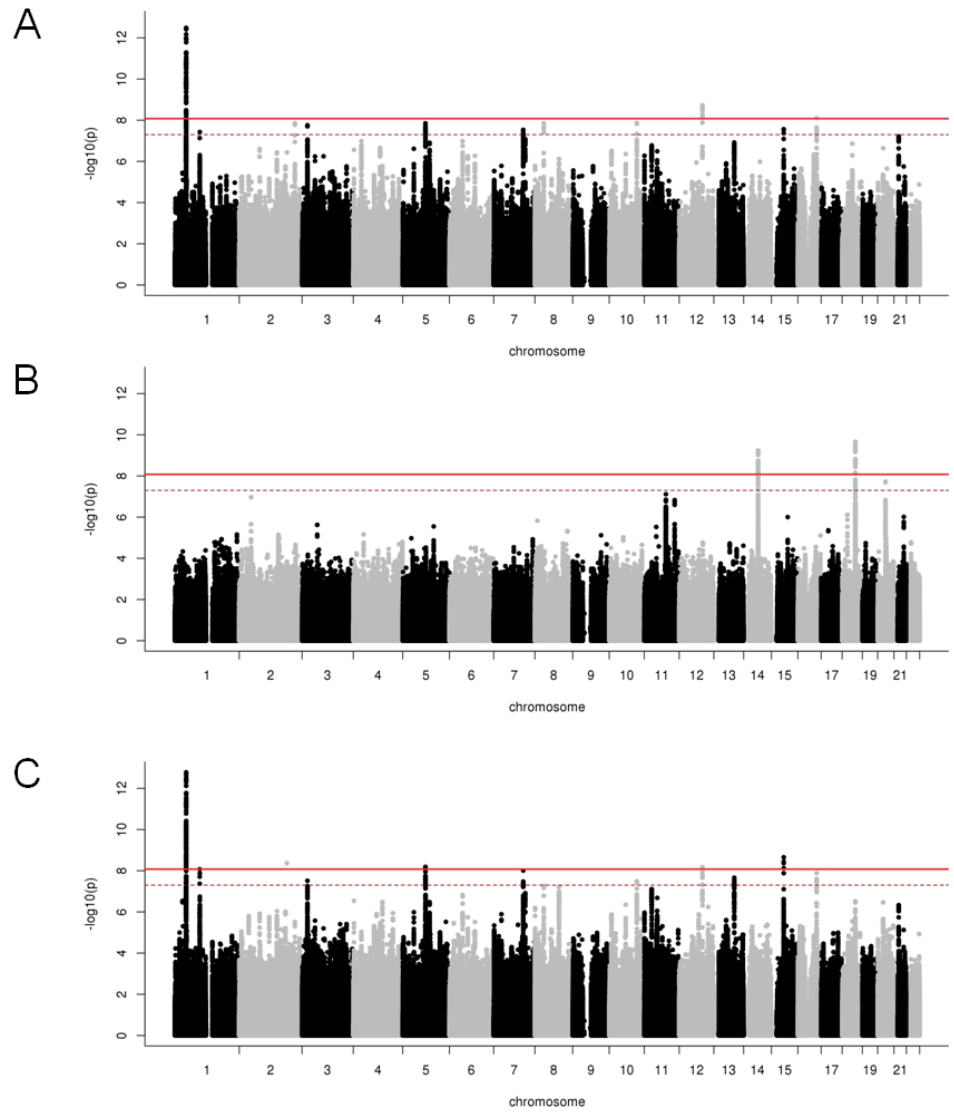
Supplementary Figure 4: Common genetic variants associated with ADHD, amygdala and the meta-analysis of ADHD and amygdala. Manhattan plots in which every point represents a single genetic variant plotted according to its genomics position (x-axis) and its $-\log_{10}(P)$ for association with the respective trait (y-axis). The solid bright red line represents the study-wide genome-wide significance of $P<8.33x10^{-9}$ and the dashed dark red line represents the genome-wide significance of $P<5x10^{-8}$. (A) PGC+iPSYCH ADHD GWAS-MA. (B) ENIGMA amygdala GWAS-MA. (C) ADHD+amygdala naive GWAS-MA.



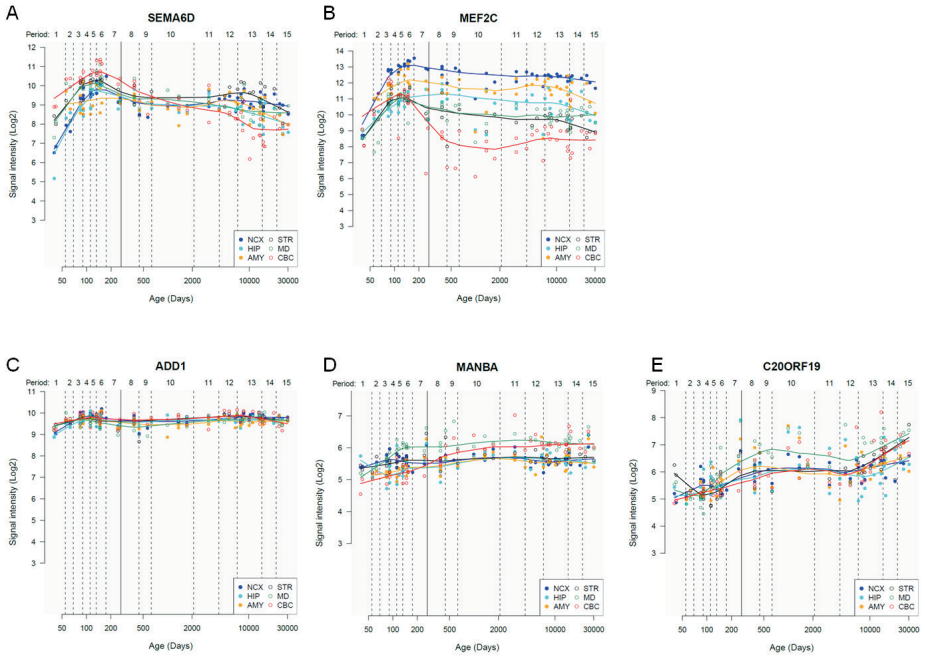
Supplementary Figure 5: Common genetic variants associated with ADHD, caudate nucleus and the meta-analysis of ADHD and caudate nucleus. Manhattan plots in which every point represents a single genetic variant plotted according to its genomics position (x-axis) and its $-\log_{10}(P)$ for association with the respective trait (y-axis). The solid bright red line represents the study-wide genome-wide significance of $P<8.33x10^{-9}$ and the dashed dark red line represents the genome-wide significance of $P<5x10^{-8}$. (A) PGC+iPSYCH ADHD GWAS-MA. (B) ENIGMA caudate nucleus GWAS-MA. (C) ADHD+caudate nucleus weighted GWAS-MA.



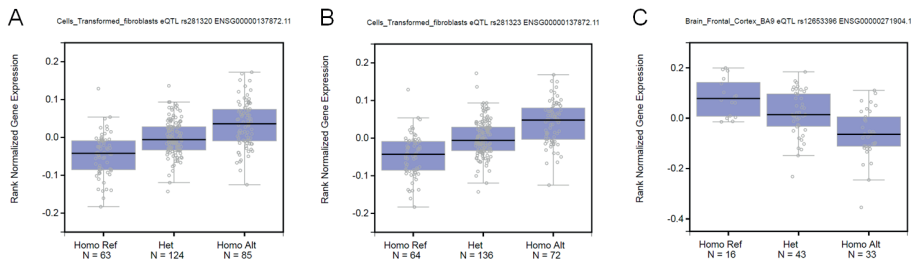
Supplementary Figure 6: Common genetic variants associated with ADHD, hippocampus and the meta-analysis of ADHD and hippocampus. Manhattan plots in which every point represents a single genetic variant plotted according to its genomics position (x-axis) and its $-\log_{10}(P)$ for association with the respective trait (y-axis). The solid bright red line represents the study-wide genome-wide significance of $P<8.33x10^{-9}$ and the dashed dark red line represents the genome-wide significance of $P<5x10^{-8}$. (A) PGC+iPSYCH ADHD GWAS-MA. (B) ENIGMA+CHARGE hippocampus GWAS-MA. (C) ADHD+hippocampus weighted GWAS-MA.



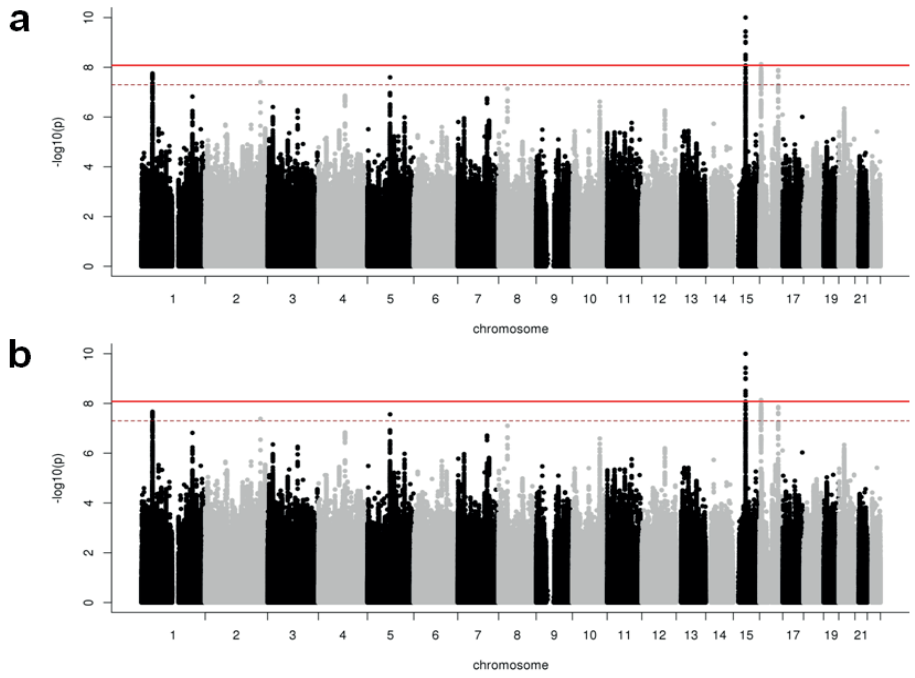
Supplementary Figure 7: Common genetic variants associated with ADHD, putamen and the meta-analysis of ADHD and putamen. Manhattan plots in which every point represents a single genetic variant plotted according to its genomics position (x-axis) and its $-\log_{10}(P)$ for association with the respective trait (y-axis). The solid bright red line represents the study-wide genome-wide significance of $P<8.33x10^{-9}$ and the dashed dark red line represents the genome-wide significance of $P<5x10^{-8}$. (A) PGC+iPSYCH ADHD GWAS-MA. (B) ENIGMA putamen GWAS-MA. (C) ADHD+putamen weighted GWAS-MA.



Supplementary Figure 8: Expression trajectories of SEMA6D (A), MEF2C (B), ADD1 (C), MANBA (D), and C20ORF19 (alias KIZ (E)) in the developing and adult human brain. Line plots show the log_2-transformed gene exon array signal intensity from the early fetal period to late adulthood in six brain regions. The solid line between periods 7 and 8 (approximately post-conception day 280) separates prenatal from postnatal periods. Data were generated using Affymetrix GeneChip Human Exon 1.0 ST Arrays by the Human Brain Transcriptome project, and accessed via their publicly available database at http://hbatlas.org (Kang et al., 2011). The *FEZF1* gene was not present in the Human Brain Transcriptome database. Abbreviations: NCX=neocortex; HIP=hippocampus; AMY=amygdala; STR=striatum; MD=mediodorsal nucleus of the thalamus; CBC=cerebellar cortex.



Supplementary Figure 9: Effect of index SNPs from ADHD+brain meta-analyses on human gene expression. (A) Expression quantitative trait loci (eQTL) analysis in transformed fibroblasts demonstrates the effect of rs281320 on *SEMA6D* gene expression. (B) eQTL analysis in transformed fibroblasts demonstrates the effect of rs281323 on *SEMA6D* gene expression. (C) eQTL analysis in frontal cortex tissue demonstrates the effect of rs12653396 on *CTC-498M16.4* gene expression. The data used here is publicly from GTEx Analysis Release V6p (GTEx Consortium, 2013).



Supplementary Figure 10: Common genetic variants associated with ADHD+ICV. Shown here are Manhattan plots, in which every point represents a single genetic variant plotted according to its genomic position (x-axis) and its $-log_{10}$(P) for association with the respective trait (y-axis). The solid bright red line represents the threshold for study-wide genome-wide significance at $P=8.33x10^{-9}$, and the dashed dark red line represents the threshold for genome-wide significance at $P=5x10^{-8}$. (**a**) ADHD+ICV weighted GWAS-MA. (**b**) ADHD+ICV naïve (without additional weight factors) GWAS-MA.

Supplementary tables

Supplementary Table 1: Sample characteristics of the different cohorts used in this study.

Cohort	Trait	$N_{subjects}$	$N_{genetic\ variants}$	Reference
PGC-iPSYCH	ADHD	20,183 cases/ 35,191 controls	8,047,420	(Demontis et al., 2017)
ENIGMA only*	Nucleus accumbens volume	11,709	8,615,659	(Hibar et al., 2015)
	Amygdala volume	11,757	8,601,199	
	Caudate nucleus volume	11,772	8,615,485	
	Hippocampus volume	11,665	8,610,806	
	Putamen volume	11,646	8,609,826	
	Intracranial volume	11,221	8,720,403	
CHARGE	Hippocampus volume	13,039	12,438,667	(Adams et al., 2016; Hibar et al., 2017)
	Intracranial volume	12,803	12,460,951	
MA ENIGMA*+CHARGE	Hippocampus volume	24,704	9,145,464	This manuscript
	Intracranial volume	24,024	9,186,920	

$N_{subjects}$=number of subjects included in this study after quality control; $N_{genetic\ variants}$=number of genetic variants available for this study after quality control. *ADHD cases from the NeuroIMAGE cohort (n=154) have been removed from this ENIGMA data set.

Supplementary Table 2. SNP heritability analyses for MRI brain volumes and genetic correlation with ADHD using constrained intercepts*.

Brain region	N	Heritability	SE	Genetic correlation with ADHD	SE	Z	P
Nucleus accumbens	11,709	0.0477	0.0315	0.144	0.1027	1.403	0.1606
Caudate nucleus	11,772	0.1714	0.0322	0.04699	0.05531	0.8495	0.3956
Hippocampus#	24,704	0.1412	0.0186	-0.01677	0.04085	-0.4104	0.6815
Intracranial volume#	24,024	0.2873	0.0229	-0.2066	0.03247	-6.363	**$1.98x10^{-10}$**
Putamen	11,646	0.1736	0.0348	0.04018	0.05257	0.7643	0.4447
Hippocampus ENIGMA only	11,665	0.1318	0.0305	-0.03867	0.05958	-0.6491	0.5163
Intracranial volume ENIGMA only	11,221	0.1809	0.0307	-0.2117	0.0555	-3.814	**0.000137**

*Amygdala mean χ^2 was too small to allow a valid analysis (N=11,757). #Using GWAS-MA summary statistics from the meta-analysis of ENIGMA and CHARGE cohorts. Heritability and genetic correlation were estimated by using constrained intercepts. P-values in bold are significant after Bonferroni correction.

Supplementary Table 3. Sign test results in brain volume cohorts.

P threshold	Brain region	N opposite direction	Proportion	P
$< 5x10^{-8}$	Nucleus accumbens	4	0.40	0.828
	Amygdala	6	0.60	0.377
	Caudate nucleus	4	0.40	0.828
	Hippocampus	4	0.40	0.828
	Intracranial volume	2	0.20	0.989
	Putamen	1	0.10	0.999
$< 1x10^{-6}$	Nucleus accumbens	14	0.40	0.912
	Amygdala	18	0.51	0.5
	Caudate nucleus	11	0.31	0.992
	Hippocampus	20	0.57	0.249
	Intracranial volume	17	0.49	0.632
	Putamen	12	0.34	0.979
$< 1x10^{-5}$	Nucleus accumbens	48	0.49	0.619
	Amygdala	52	0.53	0.307
	Caudate nucleus	38	0.39	0.990
	Hippocampus	57	0.58	0.065
	Intracranial volume	56	0.57	0.094
	Putamen	48	0.49	0.619
$< 5x10^{-8}$	Hippocampus ENIGMA only	6	0.60	0.377
	Intracranial volume ENIGMA only	3	0.33	0.910
$< 1x10^{-6}$	Hippocampus ENIGMA only	17	0.49	0.632
	Intracranial volume ENIGMA only	19	0.56	0.924
$< 1x10^{-5}$	Hippocampus ENIGMA only	50	0.51	0.459
	Intracranial volume ENIGMA only	50	0.51	0.419

Test of whether the proportion of index SNPs with estimated effects in the *opposite* direction as the ADHD GWAS-MA is greater than expected by chance. The expected proportion under the null hypothesis is 0.5. At threshold $P<5x10^{-8}$ 4 index SNPs were not available in the brain volume GWAS-MA data, so were 9 at threshold $P<5x10^{-6}$ and 34 at threshold $P<5x10^{-5}$. We set a Bonferroni-corrected significance level at P=0.05/(3*6)=0.0027.

Supplementary Table 4: Sign test results for the intelligence GWAS-MA summary statistics from (Savage et al., 2018).

P threshold	Trait	Expected directionality	N SNPs included	N expected direction	Proportion	P
< $1x10^{-5}$	ADHD+ICV	concordant	43	13	0.3023256	0.9973
		discordant	43	30	0.6976744	0.006859*
	ADHD	discordant	45	15	0.3333	0.992
	ICV	concordant	58	19	0.3275862	0.9973
< $1x10^{-4}$	ADHD+ICV	concordant	225	96	0.4266667	0.9884
		discordant	225	129	0.5733333	0.01633
	ADHD	discordant	234	133	0.5683761	0.02124
	ICV	concordant	289	120	0.4152249	0.9984

Test of whether the proportion of index SNPs with estimated effects in the *same* (concordant) or *opposite* (discordant) direction as the ADHD GWAS-MA is greater than expected by chance. This test was done for LD-independent SNPs, which (1) passed the p-value threshold of $P<1x10^{-5}$ or $P<1x10^{-4}$ in the ADHD+ICV GWAS-MA and (2) showed smaller p-value in the ADHD+ICV GWAS-MA compared to the ADHD and ICV GWAS-MA individually. The expected proportion under the null hypothesis is 0.5. We set a Bonferroni-corrected significance level at P=0.05/(2*3)=0.00833; significant results are indicated by an asterisk (*).

Supplementary Table 5: Results of MAGMA gene-based associations of all genes for ADHD and amygdala volume.

GENE	CHR	START	STOP	NSNPS	NPARAM	N	ZSTAT	P
6487	1	44073204	44496837	786	61	67131	7.2295	2.42E-13
5792	1	43891708	44189343	521	60	67131	7.1726	3.68E-13
23334	1	43755556	44019918	338	68	67131	6.9528	1.79E-12
9682	1	44015797	44271189	533	43	67131	6.693	1.09E-11
81888	1	43816674	44019938	272	57	67131	6.6747	1.24E-11
1848	12	89641837	89846296	419	96	67131	6.5685	2.54E-11
4208	5	87914058	88299922	558	85	67131	6.3061	1.43E-10
64834	1	43729068	43933745	266	53	67131	6.2814	1.68E-10
991	1	43724626	43928874	269	53	67131	6.2606	1.92E-10
4352	1	43703475	43920135	297	53	67131	6.1003	5.3E-10
112950	1	43749579	43955483	247	67	67131	6.0066	9.47E-10
80031	15	47376403	48166420	1966	166	67131	5.7416	4.69E-09
22986	10	1.06E+08	1.07E+08	2208	143	67131	5.7131	5.55E-09
7075	1	43666566	43888781	372	91	67131	5.5548	1.39E-08
9048	1	44298992	44502912	365	60	67131	5.464	2.33E-08
9670	1	44312478	44533694	417	82	67131	5.3459	4.5E-08
1006	16	61581169	62170939	1289	89	67131	5.1894	1.05E-07
389549	7	1.22E+08	1.22E+08	421	74	67131	5.1439	1.35E-07
149466	1	43647554	43851334	394	88	67131	5.1317	1.44E-07
93986	7	1.14E+08	1.14E+08	1141	150	67131	5.0418	2.31E-07
1802	1	44335653	44539043	396	72	67131	5.0381	2.35E-07
4126	4	1.03E+08	1.04E+08	572	65	67131	5.0236	2.54E-07
533	1	44340118	44543972	408	77	67131	4.9115	4.52E-07
128218	1	43635665	43839673	436	84	67131	4.8355	6.64E-07
8704	1	44344874	44556843	428	94	67131	4.8147	7.37E-07
…	…	…	…	…	…	…	…	…

Genome-wide gene-based results of MAGMA (de Leeuw et al., 2015) analysis. Entrez-ID (GENE), Chromosome (CHR), Start (START) and end (STOP) position of the genes, number of SNPs in the genes (N SNPs), effective number of SNPs included (NPARAM), the total sample size (N), test statistics (ZSTAT), and gene-based p-values for ADHD GWAS-MA (tab 1), ENIGMA amygdala GWAS-MA (tab 2), and the weighted ADHD+amygdala GWAS-MA (tab 3) are shown. Genes were considered gene-wide significant, if they reached the Bonferroni correction threshold adjusted for the total number of genes (N=18,306; $P<2.731x10^{-6}$; genes marked in bold).

*The complete **Supplementary Table 5** is available upon request.*

Supplementary Table 6: Results of MAGMA gene-based associations of all genes for ADHD and nucleus accumbens volume.

GENE	CHR	START	STOP	NSNPS	NPARAM	N	ZSTAT	P
6487	1	44073204	44496837	786	61	67083	7.1157	5.57E-13
5792	1	43891708	44189343	521	60	67083	7.0727	7.6E-13
23334	1	43755556	44019918	338	68	67083	6.8405	3.95E-12
1848	12	89641837	89846296	419	96	67083	6.6496	1.47E-11
9682	1	44015797	44271189	533	43	67083	6.6225	1.77E-11
81888	1	43816674	44019938	272	57	67083	6.5592	2.71E-11
4208	5	87914058	88299922	558	85	67083	6.2858	1.63E-10
64834	1	43729068	43933745	266	53	67083	6.1636	3.55E-10
991	1	43724626	43928874	269	53	67083	6.1406	4.11E-10
4352	1	43703475	43920135	297	53	67083	5.9543	1.31E-09
80031	15	47376403	48166420	1966	166	67083	5.9187	1.62E-09
112950	1	43749579	43955483	247	67	67083	5.9108	1.7E-09
7075	1	43666566	43888781	372	91	67083	5.5191	1.7E-08
22986	10	1.06E+08	1.07E+08	2208	143	67083	5.4726	2.22E-08
93986	7	1.14E+08	1.14E+08	1141	150	67083	5.3608	4.14E-08
1006	16	61581169	62170939	1289	89	67083	5.3444	4.54E-08
9048	1	44298992	44502912	365	60	67083	5.3367	4.73E-08
9670	1	44312478	44533694	417	82	67083	5.2546	7.42E-08
389549	7	1.22E+08	1.22E+08	421	74	67083	5.2087	9.51E-08
149466	1	43647554	43851334	394	88	67083	5.0882	1.81E-07
1802	1	44335653	44539043	396	72	67083	4.9823	3.14E-07
128218	1	43635665	43839673	436	84	67083	4.8499	6.17E-07
533	1	44340118	44543972	408	77	67083	4.8473	6.26E-07
4126	4	1.03E+08	1.04E+08	572	65	67083	4.8401	6.49E-07
3769	2	2.34E+08	2.34E+08	486	42	67083	4.8298	6.83E-07
…	…	…	…	…	…	…	…	…

Genome-wide gene-based results of MAGMA (de Leeuw et al., 2015) analysis. Entrez-ID (GENE), Chromosome (CHR), Start (START) and end (STOP) position of the genes, number of SNPs in the genes (N SNPs), effective number of SNPs included (NPARAM), the total sample size (N), test statistics (ZSTAT), and gene-based p-values for ADHD GWAS-MA (tab 1), ENIGMA nucleus accumbens GWAS-MA (tab 2), and the weighted ADHD+nucleus accumbens GWAS-MA (tab 3) are shown. Genes were considered gene-wide significant, if they reached the Bonferroni correction threshold adjusted for the total number of genes (N=18,306; $P<2.731x10^{-6}$; genes marked in bold).

*The complete **Supplementary Table 6** is available upon request.*

Supplementary Table 7: Results of MAGMA gene-based associations of all genes for ADHD and caudate nucleus volume.

GENE	CHR	START	STOP	NSNPS	NPARAM	N	ZSTAT	P
6487	1	44073204	44496837	786	61	67146	6.949	1.84E-12
5792	1	43891708	44189343	521	60	67146	6.863	3.37E-12
23334	1	43755556	44019918	338	68	67146	6.663	1.34E-11
1848	12	89641837	89846296	419	96	67146	6.5652	2.6E-11
4208	5	87914058	88299922	558	85	67146	6.4967	4.11E-11
9682	1	44015797	44271189	533	43	67146	6.4939	4.18E-11
81888	1	43816674	44019938	272	57	67146	6.3903	8.28E-11
64834	1	43729068	43933745	266	53	67146	5.9628	1.24E-09
991	1	43724626	43928874	269	53	67146	5.9289	1.52E-09
112950	1	43749579	43955483	247	67	67146	5.7776	3.79E-09
4352	1	43703475	43920135	297	53	67146	5.6684	7.21E-09
1006	16	61581169	62170939	1289	89	67146	5.406	3.22E-08
389549	7	1.22E+08	1.22E+08	421	74	67146	5.3697	3.94E-08
7075	1	43666566	43888781	372	91	67146	5.2062	9.64E-08
22986	10	1.06E+08	1.07E+08	2208	143	67146	5.2059	9.65E-08
93986	7	1.14E+08	1.14E+08	1141	150	67146	5.1629	1.22E-07
9048	1	44298992	44502912	365	60	67146	5.077	1.92E-07
151648	3	20102085	20327724	850	71	67146	4.9298	4.11E-07
118	4	2745454	3031803	770	146	67146	4.9086	4.59E-07
9670	1	44312478	44533694	417	82	67146	4.9029	4.72E-07
8850	3	19981524	20295896	1066	89	67146	4.8604	5.86E-07
3769	2	2.34E+08	2.34E+08	486	42	67146	4.852	6.11E-07
80031	15	47376403	48166420	1966	166	67146	4.8249	7E-07
149466	1	43647554	43851334	394	88	67146	4.7758	8.95E-07
…	…	…	…	…	…	…	…	…

Genome-wide gene-based results of MAGMA (de Leeuw et al., 2015) analysis. Entrez-ID (GENE), Chromosome (CHR), Start (START) and end (STOP) position of the genes, number of SNPs in the genes (N SNPs), effective number of SNPs included (NPARAM), the total sample size (N), test statistics (ZSTAT), and gene-based p-values for ADHD GWAS-MA (tab 1), ENIGMA caudate nucleus GWAS-MA (tab 2), and the weighted ADHD+caudate nucleus GWAS-MA (tab 3) are shown. Genes were considered gene-wide significant, if they reached the Bonferroni correction threshold adjusted for the total number of genes (N=18,306; $P<2.731x10^{-6}$; genes marked in bold).

*The complete **Supplementary Table 7** is available upon request.*

Supplementary Table 8: Results of MAGMA gene-based associations of all genes for ADHD and hippocampus volume.

GENE	CHR	START	STOP	NSNPS	NPARAM	N	ZSTAT	P
6487	1	44073204	44496837	796	64	67039	7.2257	2.49E-13
5792	1	43891708	44189343	522	60	67039	7.1666	3.85E-13
9682	1	44015797	44271189	534	45	67039	6.8439	3.85E-12
23334	1	43755556	44019918	339	69	67039	6.5507	2.86E-11
81888	1	43816674	44019938	272	57	67039	6.3541	1.05E-10
4126	4	1.03E+08	1.04E+08	577	67	67039	5.9974	1E-09
29979	9	86174878	86423168	478	57	67039	5.7392	4.76E-09
991	1	43724626	43928874	271	59	67039	5.7224	5.25E-09
64834	1	43729068	43933745	267	56	67039	5.7073	5.74E-09
22986	10	1.06E+08	1.07E+08	2225	147	67039	5.6764	6.88E-09
23245	9	1.19E+08	1.2E+08	3253	433	67039	5.6558	7.76E-09
4790	4	1.03E+08	1.04E+08	604	78	67039	5.5479	1.45E-08
112950	1	43749579	43955483	248	68	67039	5.5059	1.84E-08
9670	1	44312478	44533694	429	87	67039	5.4926	1.98E-08
4352	1	43703475	43920135	299	52	67039	5.4338	2.76E-08
414328	9	86137964	86359045	433	72	67039	5.3148	5.34E-08
9048	1	44298992	44502912	376	64	67039	5.3105	5.47E-08
1848	12	89641837	89846296	425	98	67039	5.2898	6.12E-08
80318	9	86254336	86544431	566	41	67039	5.2093	9.48E-08
7075	1	43666566	43888781	376	93	67039	5.1493	1.31E-07
1802	1	44335653	44539043	405	78	67039	5.1296	1.45E-07
80031	15	47376403	48166420	1978	169	67039	5.0847	1.84E-07
57497	6	40259373	40655126	1369	138	67039	5.0741	1.95E-07
1006	16	61581169	62170939	1298	93	67039	5.0376	2.36E-07
…	…	…	…	…	…	…	…	…

Genome-wide gene-based results of MAGMA (de Leeuw et al., 2015) analysis. Entrez-ID (GENE), Chromosome (CHR), Start (START) and end (STOP) position of the genes, number of SNPs in the genes (N SNPs), effective number of SNPs included (NPARAM), the total sample size (N), test statistics (ZSTAT), and gene-based p-values for ADHD GWAS-MA (tab 1), ENIGMA+CHARGE hippocampus GWAS-MA (tab 2), and the weighted ADHD+hippocampus GWAS-MA (tab 3) are shown. Genes were considered gene-wide significant, if they reached the Bonferroni correction threshold adjusted for the total number of genes (N=18,306; $P<2.731x10^{-6}$; genes marked in bold).

*The complete **Supplementary Table 8** is available upon request.*

Supplementary Table 9: Results of MAGMA gene-based associations of all genes for ADHD and putamen volume.

GENE	CHR	START	STOP	NSNPS	NPARAM	N	ZSTAT	P
6487	1	44073204	44496837	786	61	67020	7.3574	9.37E-14
5792	1	43891708	44189343	521	60	67020	7.1283	5.08E-13
9682	1	44015797	44271189	533	43	67020	6.7655	6.64E-12
23334	1	43755556	44019918	338	68	67020	6.7526	7.26E-12
81888	1	43816674	44019938	272	57	67020	6.4666	5.01E-11
4208	5	87914058	88299922	558	85	67020	6.4559	5.38E-11
1848	12	89641837	89846296	419	96	67020	6.385	8.57E-11
64834	1	43729068	43933745	266	53	67020	6.0917	5.58E-10
991	1	43724626	43928874	269	53	67020	6.0624	6.71E-10
80031	15	47376403	48166420	1966	166	67020	5.9544	1.31E-09
112950	1	43749579	43955483	247	67	67020	5.8549	2.39E-09
4352	1	43703475	43920135	297	53	67020	5.8423	2.57E-09
9048	1	44298992	44502912	365	60	67020	5.5363	1.54E-08
9670	1	44312478	44533694	417	82	67020	5.3978	3.37E-08
7075	1	43666566	43888781	372	91	67020	5.3776	3.78E-08
1630	18	49766542	51162273	4557	227	67020	5.2921	6.05E-08
22986	10	1.06E+08	1.07E+08	2208	143	67020	5.1791	1.11E-07
55857	20	21006624	21327260	521	55	67020	5.1203	1.53E-07
1802	1	44335653	44539043	396	72	67020	5.074	1.95E-07
149466	1	43647554	43851334	394	88	67020	4.9879	3.05E-07
533	1	44340118	44543972	408	77	67020	4.9292	4.13E-07
8874	13	1.12E+08	1.12E+08	1317	176	67020	4.8806	5.29E-07
93986	7	1.14E+08	1.14E+08	1141	150	67020	4.8668	5.67E-07
389549	7	1.22E+08	1.22E+08	421	74	67020	4.8493	6.2E-07
…	…	…	…	…	…	…	…	…

Genome-wide gene-based results of MAGMA (de Leeuw et al., 2015) analysis. Entrez-ID (GENE), Chromosome (CHR), Start (START) and end (STOP) position of the genes, number of SNPs in the genes (N SNPs), effective number of SNPs included (NPARAM), the total sample size (N), test statistics (ZSTAT), and gene-based p-values for ADHD GWAS-MA (tab 1), ENIGMA putamen GWAS-MA (tab 2), and the weighted ADHD+putamen GWAS-MA (tab 3) are shown. Genes were considered gene-wide significant, if they reached the Bonferroni correction threshold adjusted for the total number of genes (N=18,306; $P<2.731x10^{-6}$; genes marked in bold).

*The complete **Supplementary Table 9** is available upon request.*

Supplementary Table 10: Results of MAGMA gene-based associations of all genes for ADHD and ICV.

GENE	CHR	START	STOP	NSNPS	NPARAM	N	ZSTAT	P
6487	1	44073204	44496837	828	74	55374	7.5192	2.755E-14
5792	1	43891708	44189343	536	65	55374	7.3208	1.2327E-13
23334	1	43755556	44019918	345	73	55374	7.0027	1.2552E-12
81888	1	43816674	44019938	277	61	55374	6.8669	3.2804E-12
9682	1	44015797	44271189	551	43	55374	6.836	4.0725E-12
1848	12	89641837	89846296	436	98	55374	6.482	4.5262E-11
991	1	43724626	43928874	279	62	55374	6.2968	1.5191E-10
64834	1	43729068	43933745	275	59	55374	6.2813	1.6783E-10
112950	1	43749579	43955483	254	48	55374	6.2262	2.389E-10
4208	5	87914058	88299922	590	86	55374	6.0806	5.9876E-10
4352	1	43703475	43920135	305	54	55374	5.8981	1.8383E-09
80031	15	47376403	48166420	2021	167	55374	5.7919	3.4794E-09
7075	1	43666566	43888781	388	96	55374	5.5932	1.1146E-08
22986	10	106300859	107124993	2283	141	55374	5.526	1.6383E-08
9670	1	44312478	44533694	446	90	55374	5.377	3.7875E-08
1006	16	61581169	62170939	1336	100	55374	5.3291	4.9347E-08
9048	1	44298992	44502912	392	64	55374	5.3135	5.3775E-08
1802	1	44335653	44539043	419	82	55374	5.1964	1.016E-07
149466	1	43647554	43851334	408	90	55374	5.0717	1.9719E-07
4126	4	103452643	103782151	601	70	55374	5.046	2.2558E-07
533	1	44340118	44543972	432	87	55374	5.0317	2.4309E-07
93986	7	113626365	114433827	1182	163	55374	4.9611	3.5042E-07
8704	1	44344874	44556843	453	106	55374	4.9178	4.3771E-07
128218	1	43635665	43839673	453	95	55374	4.8618	5.8163E-07
389549	7	121841373	122051173	467	91	55374	4.8556	6.0003E-07
…	…	…	…	…	…	…	…	…

Genome-wide gene-based results of MAGMA (de Leeuw et al., 2015) analysis. Entrez-ID (GENE), Chromosome (CHR), Start (START) and end (STOP) position of the genes, number of SNPs in the genes (N SNPs), effective number of SNPs included (NPARAM), the total sample size (N), test statistics (ZSTAT), and gene-based p-values for ADHD GWAS-MA (tab 1), ENIGMA+CHARGE ICV GWAS-MA (tab 2), and the weighted ADHD+ICV GWAS-MA (tab 3) are shown. Genes were considered gene-wide significant, if they reached the Bonferroni correction threshold adjusted for the total number of genes (N=18,306; $P<2.731x10^{-6}$; genes marked in bold).

*The complete **Supplementary Table 10** is available upon request.*

Supplementary Table 11: Comparison of genome-wide significant MAGMA gene-based results for ADHD and ICV.

Gene Name	EntrezID	P_{ADHD}	P_{ICV}	$P_{ADHD+ICV}$
SEMA6D	80031	$3.48x10^{-09}$	0.002926	**$1.84x10^{-12}$**
MEF2C	4208	$5.99x10^{-10}$	0.001512	**$2.49x10^{-10}$**
PTPRF	5792	$1.23x10^{-13}$	0.55807	$7.37x10^{-09}$
SZT2	23334	$1.26x10^{-12}$	0.81234	$1.41x10^{-08}$
KIZ	55857	$6.47x10^{-07}$	0.015378	**$1.48x10^{-08}$**
DUSP6	1848	$4.53x10^{-11}$	0.73979	$4.73x10^{-08}$
HYI	81888	$3.28x10^{-12}$	0.74378	$8.07x10^{-08}$
KDM4A	9682	$4.07x10^{-12}$	0.348	$8.10x10^{-08}$
CDC20	991	$1.52x10^{-10}$	0.7465	$1.73x10^{-07}$
ELOVL1	64834	$1.68x10^{-10}$	0.74685	$1.96x10^{-07}$
MPL	4352	$1.84x10^{-09}$	0.62443	$4.45x10^{-07}$
MED8	112950	$2.39x10^{-10}$	0.73188	$7.98x10^{-07}$
TIE1	7075	$1.11x10^{-08}$	0.72988	$1.42x10^{-06}$
ST3GAL3	6487	$2.76x10^{-14}$	0.068167	$1.50x10^{-06}$
RUNX1T1	862	$1.28x10^{-06}$	0.43967	$2.05x10^{-06}$
FOXP2	93986	$3.50x10^{-07}$	0.51984	$2.67x10^{-06}$

Genome-wide significant gene-based results of MAGMA (de Leeuw et al., 2015) for the 16 genes overlapping between the ADHD and ADHD+ICV data sets. Three genes showed stronger association (smaller cross-trait p-value and nominally significant p-value ($P<0.05$) in ICV data set, marked in bold) in the cross-trait meta-analysis compared to the separate analyses of ADHD and ICV.

Supplementary Table 12: Comparison of genome-wide significant MAGMA gene-based results for ADHD and amygdala volume.

Gene name	EntrezID	P_{ADHD}	$P_{amygdala}$	$P_{ADHD+amygdala}$
ST3GAL3	6487	2.76E-14	0.25993	2.42E-13
PTPRF	5792	1.23E-13	0.23627	3.68E-13
SZT2	23334	1.26E-12	0.85979	1.79E-12
KDM4A	9682	4.07E-12	0.094323	1.09E-11
HYI	81888	3.28E-12	0.83715	1.24E-11
DUSP6	1848	4.53E-11	0.54697	2.54E-11
MEF2C	4208	5.99E-10	0.61052	1.43E-10
ELOVL1	64834	1.68E-10	0.83649	1.68E-10
CDC20	991	1.52E-10	0.83847	1.92E-10
MPL	4352	1.84E-09	0.82465	5.30E-10
MED8	112950	2.39E-10	0.82975	9.47E-10
SEMA6D	80031	3.48E-09	0.59009	4.69E-09
SORCS3	22986	1.64E-08	0.26674	5.55E-09
TIE1	7075	1.11E-08	0.65502	1.39E-08
ARTN	9048	5.38E-08	0.72927	2.33E-08
IPO13	9670	3.79E-08	0.87871	4.50E-08
CDH8	1006	4.93E-08	0.17713	1.05E-07
FEZF1	389549	6.00E-07	0.017209	**1.35E-07**
C1ORF210	149466	1.97E-07	0.59275	1.44E-07
FOXP2	93986	3.50E-07	0.75094	2.31E-07
DPH2	1802	1.02E-07	0.89686	2.35E-07
MANBA	4126	2.26E-07	0.26696	2.54E-07
ATP6V0B	533	2.43E-07	0.91025	4.52E-07
TMEM125	128218	5.82E-07	0.48427	6.64E-07
B4GALT2	8704	4.38E-07	0.89299	7.37E-07
KIZ	55857	6.47E-07	0.2255	7.65E-07
POC1B	282809	1.09E-06	0.75771	9.53E-07
ADD1	118	1.24E-06	0.83043	1.07E-06
RUNX1T1	862	1.28E-06	0.041461	1.41E-06

Genome-wide significant gene-based results of MAGMA (de Leeuw et al., 2015) for the 29 genes overlapping between the ADHD and ADHD+amygdala data sets. One gene showed stronger association (smaller cross-trait p-value and nominally significant p-value (P<0.05) in amygdala data set, marked in bold) in the cross-trait meta-analysis compared to the separate analyses of ADHD and amygdala.

Supplementary Table 13: Comparison of genome-wide significant MAGMA gene-based results for ADHD and nucleus accumbens volume.

Gene name	**EntrezID**	**P_{ADHD}**	**$P_{accumbens}$**	**$P_{ADHD+accumbens}$**
ST3GAL3	6487	2.755E-14	0.94551	5.5663E-13
PTPRF	5792	1.2327E-13	0.94222	7.5999E-13
SZT2	23334	1.2552E-12	0.87531	3.945E-12
DUSP6	1848	4.5262E-11	0.43645	1.4698E-11
KDM4A	9682	4.0725E-12	0.96018	1.7656E-11
HYI	81888	3.2804E-12	0.86699	2.7053E-11
MEF2C	4208	5.9876E-10	0.63371	1.6309E-10
ELOVL1	64834	1.6783E-10	0.8459	3.5548E-10
CDC20	991	1.5191E-10	0.83473	4.1098E-10
MPL	4352	1.8383E-09	0.74892	1.306E-09
SEMA6D	80031	3.4794E-09	0.26938	1.6221E-09
MED8	112950	2.389E-10	0.90161	1.7026E-09
TIE1	7075	1.1146E-08	0.36207	1.7039E-08
SORCS3	22986	1.6383E-08	0.22952	2.2168E-08
FOXP2	93986	3.5042E-07	0.39951	4.1417E-08
CDH8	1006	4.9347E-08	0.74346	4.5358E-08
ARTN	9048	5.3775E-08	0.76902	4.7319E-08
IPO13	9670	3.7875E-08	0.6043	7.4177E-08
FEZF1	389549	6.0003E-07	0.66124	9.5065E-08
C1ORF210	149466	1.9719E-07	0.27701	1.8071E-07
DPH2	1802	1.016E-07	0.60244	3.1423E-07
TMEM125	128218	5.8163E-07	0.17473	6.1746E-07
ATP6V0B	533	2.4309E-07	0.56608	6.2568E-07
MANBA	4126	2.2558E-07	0.89609	6.4895E-07
ADD1	118	1.2405E-06	0.30503	9.2319E-07
B4GALT2	8704	4.3771E-07	0.52686	1.0343E-06
KIZ	55857	6.4723E-07	0.91178	1.1743E-06
POC1B	282809	1.0927E-06	0.2469	1.4311E-06
LRFN2	57497	2.5802E-06	0.10434	1.8371E-06
CEND1	51286	2.4283E-06	0.85151	2.1283E-06

Genome-wide significant gene-based results of MAGMA (de Leeuw et al., 2015) for the 30 genes overlapping between the ADHD and ADHD+nucleus accumbens data sets. No gene showed stronger association (smaller cross-trait p-value and nominally significant p-value (P<0.05) in nucleus accumbens data set) in the cross-trait meta-analysis compared to the separate analyses of ADHD and nucleus accumbens.

Supplementary Table 14: Comparison of genome-wide significant MAGMA gene-based results for ADHD and caudate nucleus volume.

Gene name	EntrezID	P_{ADHD}	$P_{caudate}$	$P_{ADHD+acaudate}$
ST3GAL3	6487	2.755E-14	0.88846	1.84E-12
PTPRF	5792	1.2327E-13	0.88716	3.3722E-12
SZT2	23334	1.2552E-12	0.93397	1.3417E-11
DUSP6	1848	4.5262E-11	0.42831	2.599E-11
MEF2C	4208	5.9876E-10	0.067713	4.1061E-11
KDM4A	9682	4.0725E-12	0.64592	4.1825E-11
HYI	81888	3.2804E-12	0.94235	8.2796E-11
ELOVL1	64834	1.6783E-10	0.85376	1.2399E-09
CDC20	991	1.5191E-10	0.85221	1.5244E-09
MED8	112950	2.389E-10	0.85312	3.7897E-09
MPL	4352	1.8383E-09	0.85695	7.2054E-09
CDH8	1006	4.9347E-08	0.47936	3.2218E-08
FEZF1	389549	6.0003E-07	0.10085	3.9442E-08
TIE1	7075	1.1146E-08	0.65191	9.637E-08
SORCS3	22986	1.6383E-08	0.80094	9.6541E-08
FOXP2	93986	3.5042E-07	0.47231	1.2157E-07
ARTN	9048	5.3775E-08	0.98566	1.9172E-07
ADD1	118	1.2405E-06	0.0162	**4.586E-07**
IPO13	9670	3.7875E-08	0.96342	4.721E-07
SEMA6D	80031	3.4794E-09	0.22968	7.0042E-07
C1ORF210	149466	1.9719E-07	0.5263	8.9503E-07
RUNX1T1	862	0.000001283	0.43918	1.0648E-06
POC1B	282809	1.0927E-06	0.45431	1.2555E-06
DPH2	1802	1.016E-07	0.93774	0.000002359
TMEM125	128218	5.8163E-07	0.33768	0.000002393

Genome-wide significant gene-based results of MAGMA (de Leeuw et al., 2015) for the 25 genes overlapping between the ADHD and ADHD+caudate nucleus data sets. One gene showed stronger association (smaller cross-trait p-value and nominally significant p-value (P<0.05) in caudate nucleus data set, marked in bold) in the cross-trait meta-analysis compared to the separate analyses of ADHD and caudate nucleus.

Supplementary Table 15: Comparison of genome-wide significant MAGMA gene-based results for ADHD and hippocampus volume.

Gene name	EntrezID	P_{ADHD}	$P_{hippocampus}$	$P_{ADHD+hippocampus}$
ST3GAL3	6487	2.76E-14	0.61499	2.49E-13
PTPRF	5792	1.23E-13	0.49516	3.85E-13
KDM4A	9682	4.07E-12	0.43352	3.85E-12
SZT2	23334	1.26E-12	0.70324	2.86E-11
HYI	81888	3.28E-12	0.59579	1.05E-10
MANBA	4126	2.26E-07	0.000875	**1.00E-09**
CDC20	991	1.52E-10	0.87975	5.25E-09
ELOVL1	64834	1.68E-10	0.88041	5.74E-09
SORCS3	22986	1.64E-08	0.41568	6.88E-09
MED8	112950	2.39E-10	0.84623	1.84E-08
IPO13	9670	3.79E-08	0.58968	1.98E-08
MPL	4352	1.84E-09	0.9076	2.76E-08
ARTN	9048	5.38E-08	0.66681	5.47E-08
DUSP6	1848	4.53E-11	0.43039	6.12E-08
TIE1	7075	1.11E-08	0.87312	1.31E-07
DPH2	1802	1.02E-07	0.34401	1.45E-07
SEMA6D	80031	3.48E-09	0.98523	1.84E-07
LRFN2	57497	2.58E-06	0.078658	1.95E-07
CDH8	1006	4.93E-08	0.40683	2.36E-07
ATP6V0B	533	2.43E-07	0.22951	3.45E-07
B4GALT2	8704	4.38E-07	0.32276	6.51E-07
C1ORF210	149466	1.97E-07	0.8807	9.91E-07
MEF2C	4208	5.99E-10	0.007951	1.14E-06
KIZ	55857	6.47E-07	0.5552	1.44E-06

Genome-wide significant gene-based results of MAGMA (de Leeuw et al., 2015) for the 24 genes overlapping between the ADHD and ADHD+hippocampus data sets. One gene showed stronger association (smaller cross-trait p-value and nominally significant p-value (P<0.05) in caudate nucleus data set, marked in bold) in the cross-trait meta-analysis compared to the separate analyses of ADHD and caudate nucleus.

Supplementary Table 16: Comparison of genome-wide significant MAGMA gene-based results for ADHD and putamen volume.

Gene name	EntrezID	P_{ADHD}	$P_{putamen}$	$P_{ADHD+putamen}$
ST3GAL3	6487	2.76E-14	0.36927	9.37E-14
PTPRF	5792	1.23E-13	0.59089	5.08E-13
KDM4A	9682	4.07E-12	0.32362	6.64E-12
SZT2	23334	1.26E-12	0.91611	7.26E-12
HYI	81888	3.28E-12	0.92929	5.01E-11
MEF2C	4208	5.99E-10	0.49053	5.38E-11
DUSP6	1848	4.53E-11	0.72256	8.57E-11
ELOVL1	64834	1.68E-10	0.8585	5.58E-10
CDC20	991	1.52E-10	0.85348	6.71E-10
SEMA6D	80031	3.48E-09	0.11999	1.31E-09
MED8	112950	2.39E-10	0.9054	2.39E-09
MPL	4352	1.84E-09	0.79741	2.57E-09
ARTN	9048	5.38E-08	0.52654	1.54E-08
IPO13	9670	3.79E-08	0.57582	3.37E-08
TIE1	7075	1.11E-08	0.73736	3.78E-08
SORCS3	22986	1.64E-08	0.57283	1.11E-07
KIZ	55857	6.47E-07	0.14818	1.53E-07
DPH2	1802	1.02E-07	0.62207	1.95E-07
C1ORF210	149466	1.97E-07	0.60083	3.05E-07
ATP6V0B	533	2.43E-07	0.64662	4.13E-07
FOXP2	93986	3.50E-07	0.61062	5.67E-07
FEZF1	389549	6.00E-07	0.72766	6.20E-07
B4GALT2	8704	4.38E-07	0.65805	6.86E-07
RUNX1T1	862	1.28E-06	0.62215	9.28E-07
TMEM125	128218	5.82E-07	0.56387	1.24E-06
CEND1	51286	2.43E-06	0.24057	2.65E-06

Genome-wide significant gene-based results of MAGMA (de Leeuw et al., 2015) for the 26 genes overlapping between the ADHD and ADHD+putamen data sets. No gene showed stronger association (smaller cross-trait p-value and nominally significant p-value (P<0.05) in putamen data set) in the cross-trait meta-analysis compared to the separate analyses of ADHD and putamen.

Supplementary Table 17. Reciprocal look-up of significantly associated ADHD index SNPs in brain volume GWAS data.

									Amygdala		Accumbens		Caudate		Hippocampus ENIGMA+CHARGE		ICV ENIGMA + CHARGE		…	
Index SNP	A1	A2	chr	pos	OR_{ADHD}	P_{ADHD}	Proxy	LD to index (r2)	Zscore	P	Zscore	P	Zscore	P	Zscore	P	Zscore	P	…	…
rs11420276	G	GT	1	44184192	1.113	2.14×10^{-13}	rs112984125	0.976	-0.19465	0.8457	-0.37425	0.7082	-0.74975	0.4534	-1.806	0.07085	**1.259**	0.2082	…	…
rs1222063	A	G	1	96602440	1.101	3.07×10^{-8}	rs1222067	0.751	-0.82901	0.4071	-0.70142	0.483	-0.04405	0.9649	**1.011**	0.3121	**0.198**	0.8432	…	…
rs2391769	A	G	1	96978961	0.927	3.96×10^{-8}	/	/	**-1.53538**	0.1247	**-1.31316**	0.1891	**-0.63118**	0.5279	1.598	0.11	0.797	0.4257	…	…
rs9677504	A	G	2	215181889	1.124	1.39×10^{-8}	/	/	-0.88997	0.3735	-0.04778	0.9619	**0.090919**	0.9276	-0.42	0.6744	**0.714**	0.4755	…	…
rs4858241	T	G	3	20669071	1.082	1.74×10^{-8}	/	/	**0.629083**	0.5293	-0.05447	0.9565	-0.24647	0.8053	**0.284**	0.7761	**0.622**	0.5338	…	…
rs28411770	T	C	4	31151456	1.09	1.15×10^{-8}	rs7674790	0.783	**0.412716**	0.6798	-1.43748	0.1506	**0.020197**	0.9839	-0.349	0.727	**0.191**	0.8486	…	…
rs4916723	A	C	5	87854395	0.926	1.58×10^{-8}	/	/	0.193704	0.8464	0.137039	0.891	**-1.97909**	0.04781	0.13	0.8967	**-0.012**	0.9902	…	…
rs304132	A	G	5	88215594	0.925	4.22×10^{-8}	/	/	0.266157	0.7901	**-0.30565**	0.7599	**-0.60425**	0.5457	**-0.087**	0.9309	**-1.779**	0.07529	…	…
rs5886709	G	GTC	7	114086133	1.079	1.66×10^{-8}	rs7795397	0.941	-0.80659	0.4199	**0.577128**	0.5639	**0.255358**	0.7984	**0.135**	0.8923	**1.226**	0.2203	…	…
rs74760947	A	G	8	34352610	0.835	1.35×10^{-8}	/	/	0.973533	0.3303	**-0.63116**	0.5279	1.30129	0.1932	2.161	0.0307	**-0.544**	0.5865	…	…
rs11591402	A	T	10	106747354	0.911	1.34×10^{-8}	/	/	**-0.74372**	0.457	**-0.27891**	0.7803	**-0.54555**	0.5854	**-1.502**	0.1331	**-0.038**	0.9697	…	…
rs1427829	A	G	12	89760744	1.083	1.82×10^{-9}	/	/	-0.66256	0.5076	**1.26033**	0.2076	**1.14794**	0.251	**0.321**	0.7485	-0.066	0.9477	…	…
rs281324	T	C	15	47754018	0.928	2.68×10^{-8}	/	/	**-0.06549**	0.9478	**-1.69739**	0.08963	**-0.71869**	0.4723	**-0.147**	0.8829	**-2.817**	0.004841	…	…
rs212178	A	G	16	72578131	0.891	7.68×10^{-9}	/	/	**-0.97496**	0.3296	**-0.43887**	0.6608	1.40607	0.1597	**-1.983**	0.04736	**-1.515**	0.1297	…	…

GWAS results from brain volume GWASs for the genome-wide significant loci identified in the ADHD GWAS. Replication is tested for the index variant from the ADHD GWAS, or for a proxy variant when the index variant is not present in the brain volume cohorts. Effects (Z-score or odds ratio [OR] with reference to allele 1 [A1]) that are sign concordant with the ADHD GWAS are indicated in bold.

*The complete **Supplementary Table 17** is available upon request.*

Supplementary Table 18. Reciprocal look-up of significantly associated brain volume index SNPs in ADHD GWAS data.

Brain trait	Index SNP	A1	A2	chr	pos	$Zscore_{brain}$	P_{brain}	Proxy	LD to index (r2)	OR_{ADHD}	P_{ADHD}
Caudate nucleus[a]	rs1318862	T	C	11	92007101	5.468	$4.56x10^{-8}$	/	/	0.98255	0.1971
	rs77956314	T	C	12	117323367	-9.530	$1.63 x10^{-21}$	/	/	**0.97307**	0.2692
	rs61921502	T	G	12	65832468	8.743	$2.26 x10^{-18}$	/	/	0.97346	0.1769
Hippocampus [b]	rs11979341	C	G	7	155797978	-6.523	$6.90 x10^{-11}$	rs4716969	0.352	**0.98847**	0.5723
	rs7020341	C	G	9	119247974	6.704	$2.03 x10^{-11}$	/	/	**1.03179**	0.02228
	rs2268894	T	C	2	162856148	-7.231	$4.78 x10^{-13}$	/	/	**0.99035**	0.4634
	rs2289881	T	G	5	66084260	-5.245	$1.56 x10^{-7}$	/	/	1.0015	0.9144
	rs945270	C	G	14	56200473	9.237	$2.54 x10^{-20}$	/	/	0.98728	0.3432
Putamen[a]	rs62097986	A	C	18	50818827	6.348	$2.18 x10^{-10}$	/	/	**1.03252**	0.01785
	rs6087771	T	C	20	30306724	5.624	$1.87 x10^{-8}$	/	/	0.98501	0.3109
	rs683250	A	G	11	83276168	-5.081	$3.75 x10^{-7}$	/	/	**0.99084**	0.5009
	rs199525	T	G	17	44847834	9.262	$2.00 x10^{-20}$	/	/	0.9659	0.04306
	rs11759026	A	G	6	126792095	-8.793	$1.45 x10^{-18}$	/	/	1.03087	0.06001
	rs2022464	A	C	6	108945370	-6.418	$1.38 x10^{-10}$	/	/	**0.97893**	0.1454
Intracranial volume[b]	rs11191683	T	G	10	105170649	6.046	$1.49 x10^{-9}$	/	/	0.98955	0.4545
	rs9811910	C	G	3	190670902	5.988	$2.12 x10^{-9}$	/	/	0.98187	0.425
	rs138074335	A	G	12	66374247	5.694	$1.24 x10^{-8}$	rs8756	1	**1.04227**	**0.001863**
	rs2195243	C	G	12	102922986	-5.260	$1.44 x10^{-7}$	/	/	1.05654	**0.001267**
Hippocampus ENIGMA only[a]	rs77956314	T	C	12	117323367	-5.626	$1.84 x10^{-8}$	/	/	**0.97307**	0.2692
	rs61921502	T	G	12	65832468	5.7331	$9.89 x10^{-9}$	/	/	0.97346	0.1769
Intracranial volume ENIGMA only[a]	rs17689882	A	G	17	43906828	-5.939	$2.85 x10^{-9}$	/	/	1.0453	0.008742

GWAS results from ADHD GWAS for the genome-wide significant loci identified in the different brain volume GWASs. Those SNPs were selected from the original publications (Adams et al., 2016; Hibar et al., 2017; Hibar et al., 2015). Replication is tested for the index variant from the brain volume GWASs, or for a proxy variant when the index variant is not present in the brain volume cohorts. Effects (Z-score or odds ratio [OR] with reference to allele 1 [A1]) that are sign concordant with the brain volume GWASs are indicated in bold. P-values in bold are significant after Bonferroni correction for testing 21 variants (P<0.00238). A proxy variant (rs8756; r^2=1; chr12:66359752, located in exon 5 of *HMGA2*) of the ICV-associated variant rs138074335 (chr12:66374247, intergenic and upstream of *HMGA2*; increasing ICV) was associated with increased risk for ADHD (OR=1.042, P=0.00186). A second variant, rs2195243 (chr12:102922986, intergenic and upstream of *IGF1*), was associated with decreased ICV and increased risk for ADHD (OR=1.0565, P=0.00127). [a] Z-scores and corresponding P-values were retrieved from the ENIGMA only brain volume GWAS-MA data excluding ADHD cases. [b] Z-scores and corresponding P-values were retrieved from the ENGIMA2(without ADHD) and CHARGE meta-analysis described in this study.

Supplementary Table 19: Single tissue eQTL results for index SNPs of relevant genome-wide significant loci in the ADHD+brain volume meta-analyses.

		Index SNP	**rs281320**	**rs281323**	**rs12653396**	**rs8756**	**rs2195243**
Tissue (N)	**Gene**		*SEMA6D*	*SEMA6D*	*CTC-498M16.4*	*HMGA2*	*CCDC53*
Cells - Transformed fibroblasts (300)	P		$1.7x10^{-20}$	$7.4 x10^{-24}$	---	$9.8x10^{-6}$	$4.8 x10^{-3}$
	NES		0.52	0.55	---	-0.134	-0.132
Amygdala (88)	P		0.8	0.9	$4.5x10^{-7}$	---	0.8
	NES		-0.0215	-0.0162	-0.543	---	0.0248
Caudate (144)	P		0.6	0.5	$1.8x10^{-4}$	---	0.2
	NES		-0.0371	-0.0397	-0.339	---	0.127
Hippocampus (111)	P		0.5	0.5	$7.4x10^{-9}$	---	0.3
	NES		0.0507	0.0509	-0.605	---	0.0951
Nucleus accumbens (130)	P		0.2	0.1	$8.6x10^{-9}$	---	0.1
	NES		0.0892	0.108	-0.466	---	0.169
Putamen (111)	P		0.6	0.3	$5.6x10^{-6}$	---	0.9
	NES		0.0339	0.0817	-0.406	---	0.0188
Cortex (136)	P		0.1	0.1	$3.2x10^{-6}$	---	1
	NES		0.133	0.141	-0.451	---	0.00248
Frontal Cortex (118)	P		0.6	0.2	$4.4x10^{-7}$	---	0.7
	NES		0.0417	0.109	-0.524	---	-0.0396
Blood (5,311)	P		---	---	$6.53x10^{-7}$ *	---	---
	Z-score		---	---	-4.97	---	---

All six index SNPs from the weighted ADHD+brain volume meta-analyses with a P<$8.33x10^{-9}$ and the two significant variants from the reciprocal lookup of genome-wide significant associations were included in the eQTL analysis. All SNPs with available data in the GTEx portal (GTEx Consortium, 2013) and the blood eQTL browser (Westra et al., 2013) are shown above. Only variant rs12653396 was present in both the GTEx portal and blood eQTL browser. N=sample size. NES=normalized effect size. *cis-eQTL for *MEF2C*.

Supplementary Table 20: Results of MAGMA gene-set analyses results for the neurite outgrowth gene-set.

GWAS-MA	**NGENES**	**BETA**	**BETA_STD**	**SE**	**COMP_P**	**SELF_P**
ADHD+Intracranial volume	45	0.367	0.0182	0.136	**0.00338**	**$1.55x10^{-6}$**
ADHD	45	0.148	0.0073	0.145	0.15391	**$5.53x10^{-9}$**
Intracranial volume	45	-0.0785	-0.00389	0.14	0.71179	0.40748

Competitive (COMP_P) and self-contained (SELF_P) results of the gene-set analysis of the neurite outgrowth gene-set performed using MAGMA (de Leeuw et al., 2015). The number of genes (N GENES), raw and semi-standardized (STD) regression coefficients, and corresponding standard error (SE) are reported. Significant results after Bonferroni correction are shown in bold.

Supplementary Table 21: Results of MAGMA gene-based associations of neurite outgrowth genes.

GENE_NAME	CHR	START	STOP	NSNPS	ZSTAT	$P_{ADHD+ICV}$	P_{ADHD}	P_{ICV}
CREB5	7	28238940	28965511	1822	3.2619	**0.000553**	0.0005475	0.074382
MMP24	20	33714539	33964804	453	2.5709	0.005071	0.0034001	0.77984
TLL2	10	98024363	98373683	898	2.4879	0.006425	0.033235	0.43003
NEDD4L	18	55611580	56168772	1607	2.3373	0.009711	0.0062839	0.86232
DNM1	9	130865634	131117528	389	2.293	0.010923	0.0014409	0.58186
ASTN2	9	119087504	120277317	3253	2.1668	0.015126	0.0001023	0.093386
NRXN1	2	50045643	51359674	3836	2.1233	0.016864	0.13173	0.33824
SUPT3H	6	44694467	45445788	2144	2.0192	0.021735	0.17377	0.066473
BMPR1B	4	95579128	96179601	1564	2.0112	0.022152	0.024176	0.82239
CSMD2	1	33879609	34731443	1845	1.77	0.038365	0.00806	0.16337
ADAMTS17	15	100411643	100982183	2451	1.4316	0.076131	0.059053	0.85152
ZNF423	16	49424515	49991830	1425	1.4178	0.078126	0.2668	0.20731
GPC6	13	93779078	95160274	3351	1.1491	0.12527	0.56497	0.016549
MYT1L	2	1692885	2435147	1924	1.1262	0.13004	0.022068	0.57042
MBOAT1	6	19999915	20312695	770	1.1239	0.13054	0.036503	0.58052
PPM1H	12	62937762	63428665	1293	1.1171	0.13197	0.24579	0.0385
EMP2	16	10522279	10774539	854	1.078	0.14052	0.10888	0.20062
MAP1B	5	71303118	71605397	630	1.0463	0.14772	0.056063	0.39159
UNC5B	10	72872292	73162635	776	0.8627	0.19415	0.27714	0.57631
NOS1	12	117545921	117899607	869	0.80518	0.21036	0.30687	0.11535
CDH13	16	82560399	83930215	7024	0.80402	0.21069	0.093966	0.91052
NUCB1	19	49303307	49526540	498	0.71	0.23885	0.071321	0.36184
SLCO3A1	15	92296938	92815665	1440	0.65899	0.25495	0.22741	0.35818
CDH23	10	73056691	73675704	1730	0.63549	0.26255	0.18278	0.98778
NXPH1	7	8373585	8892593	1664	0.53694	0.29565	0.0776	0.66363
KCNIP4	4	20630234	22050424	4489	0.40482	0.34281	0.41226	0.71094
MAN2A2	15	91347420	91565815	588	0.37292	0.3546	0.29083	0.15025
HKDC1	10	70880059	71127315	687	0.30449	0.38038	0.7865	0.25629
CTNNA2	2	79640060	80975993	3424	0.23372	0.4076	0.06039	0.44387
FAM190A	4	90948684	92623370	4371	0.14582	0.44203	0.053733	0.37609
FLNC	7	128370436	128599328	468	0.075226	0.47002	0.37628	0.79661
HK1	10	70929740	71261638	1025	0.061092	0.47564	0.51193	0.52288
KCP	7	128416919	128650773	446	-0.12722	0.55062	0.6579	0.79675
SPOCK3	4	167554535	168255741	1629	-0.14745	0.55861	0.51736	0.60259
DYNC2H1	11	102880160	103450591	2201	-0.31828	0.62487	0.78806	0.70885
FHIT	3	59635036	61337133	6164	-0.36753	0.64339	0.12672	0.8485

Suppplementary Table 21: Continued.

GENE_NAME	CHR	START	STOP	NSNPS	ZSTAT	$P_{ADHD+ICV}$	P_{ADHD}	P_{ICV}
NCKAP5	2	133329361	134499118	2564	-0.37607	0.64657	0.17621	0.88675
DUSP1	5	172095093	172298203	605	-0.46873	0.68037	0.30004	0.72951
ATP2C2	16	84302129	84597793	1667	-0.48023	0.68447	0.85789	0.75574
MOBP	3	39409064	39670988	815	-0.67089	0.74885	0.83675	0.88765
ITGA11	15	68491128	68824502	751	-0.68035	0.75186	0.52294	0.37697
MEIS1	2	66562257	66899891	728	-0.96337	0.83232	0.77393	0.25917
RORA	15	60680483	61621502	2214	-1.0152	0.84501	0.81967	0.94611
UGT1A9	2	234480544	234781951	949	-1.2084	0.88654	0.30306	0.37333
LRP1B	2	140888996	142989270	7745	-1.3082	0.90461	0.91812	0.1159

MAGMA (de Leeuw et al., 2015) gene-based analysis of previously reported neurite outgrowth candidate genes (Poelmans et al., 2011). Chromosome (CHR), Start (START) and end (STOP) position of the genes, number of SNPs in the genes (N SNPs), test statistics (ZSTAT), and gene-based p-values for 1) the weighted ADHD+ICV GWAS-MA ($\mathbf{P_{ADHD+ICV}}$), 2) ADHD GWAS-MA ($\mathbf{P_{ADHD}}$), and 3) ENIGMA+CHARGE ICV GWAS-MA ($\mathbf{P_{ICV}}$) are shown. For the results of the weighted ADHD+ICV GWAS-MA, genes were considered gene-wide significant, if they reached the Bonferroni correction threshold adjusted for the number of genes within the total gene-set (N=45; P<0.00111; genes marked in bold).

Supplementary Table 22. Results for the 19 most strongly associated SNPs from the weighted ADHD+ICV GWAS-MA. Results for the naïve (non-weighted) GWAS-MA are shown in **Zscore** $_{\text{naive MA}}$ and **P** $_{\text{naive MA}}$.

SNP	CHR	BP	Zscore$_{PGC}$	Zscore$_{ICV}$	Zscore$_{naive\ MA}$	Zscore$_{weighted\ MA}$	P$_{naive\ MA}$	P$_{weighted\ MA}$
rs281320	15	47769424	-5.54867	-3.33	-6.46553	-6.4677	$1.01x10^{-10}$	$9.95x10^{-11}$
rs8039398	15	47730870	-5.48151	-3.07	-6.26643	-6.27022	$3.69x10^{-10}$	$3.61x10^{-10}$
rs1656604	15	47794252	-5.36599	-3.243	-6.26511	-6.26704	$3.73x10^{-10}$	$3.68x10^{-10}$
rs281324	15	47754018	-5.5595	-2.817	-6.19239	-6.19846	$5.93x10^{-10}$	$5.70x10^{-10}$
rs281323	15	47754027	5.477548	2.796	6.112398	6.118231	$9.81x10^{-10}$	$9.46x10^{-10}$
rs1610098	15	47806012	-5.13664	-3.297	-6.10329	-6.10369	$1.04x10^{-9}$	$1.04x10^{-9}$
rs1612378	15	47813991	-4.94219	-3.269	-5.9255	-5.92516	$3.11x10^{-9}$	$3.12x10^{-9}$
rs1656622	15	47813909	-4.94219	-3.2	-5.88754	-5.88772	$3.92x10^{-9}$	$3.92x10^{-9}$
rs1347469	15	47814528	-4.93526	-3.147	-5.8526	-5.85314	$4.84x10^{-9}$	$4.82x10^{-9}$
rs13332522	16	5829204	4.616228	3.508	5.784743	5.781012	$7.26x10^{-9}$	$7.43x10^{-9}$
rs4597332	16	5829191	-4.61653	-3.499	-5.78004	-5.77638	$7.47x10^{-9}$	$7.63x10^{-9}$
rs4513101	16	5829196	4.602781	3.505	5.771863	5.768088	$7.84x10^{-9}$	$8.02x10^{-9}$
rs1656623	15	47815484	4.820448	3.153	5.760016	5.75995	$8.41x10^{-9}$	$8.41x10^{-9}$
rs1618196	15	47797832	-4.90586	-2.985	-5.73893	-5.74055	$9.53x10^{-9}$	$9.44x10^{-9}$
rs7198618	16	5829440	4.52717	3.519	5.71642	5.71217	$1.09x10^{-8}$	$1.12x10^{-8}$
rs1656618	15	47810363	4.746723	3.133	5.687446	5.687169	$1.29x10^{-8}$	$1.29x10^{-8}$
rs12596294	16	72587093	5.572012	1.849	5.673685	5.686953	$1.40x10^{-8}$	$1.29x10^{-8}$
rs118613 10	16	5835841	4.595736	3.348	5.679619	5.676988	$1.35x10^{-8}$	$1.37x10^{-8}$
rs212178	16	72578131	-5.76998	-1.515	-5.65614	-5.67288	$1.55x10^{-8}$	$1.40x10^{-8}$

Supplementary Table 23: Overview of metadata for all individual traits and meta-analyzed summary statistics.

	ADHD	ICV*	Amygdala	Accumbens	Caudate	Hippocampus*	Putamen	ADHD+ ICV*	ADHD+ Amygdala	ADHD+ Accumbens	ADHD+ caudate	ADHD+ hippocampus*	ADHD+ putamen
Total Observed scale h2 (se)	0.234 (0.0154)	0.2348 (0.0313)	-0.0172 (0.0397)	0.1349 (0.0504)	0.2469 (0.0459)	0.1368 (0.027)	0.2922 (0.0555)	0.1112 (0.0089)	0.1883 (0.0124)	0.1827 (0.0121)	0.17 (0.0115)	0.1366 (0.0099)	0.1724 (0.0121)
Lambda GC	1.2531	1.1082	0.9986	1.0075	1.0345	1.0588	1.0345	1.1876	1.2498	1.2498	1.2332	1.2234	1.2365
Mean Chi^2	1.2973	1.1416	1.0003	1.0122	1.0415	1.0736	1.0436	1.2132	1.2973	1.2914	1.2733	1.2576	1.2745
Intercept (se)	1.0363 (0.0102)	1.0231 (0.01)	1.0046 (0.0067)	0.9785 (0.0083)	0.9818 (0.0074)	1.0024 (0.0091)	0.9719 (0.0086)	1.0284 (0.0094)	1.0333 (0.0103)	1.0344 (0.0102)	1.0351 (0.0098)	1.029 (0.0096)	1.0303 (0.0101)
Ratio (se)	0.1222 (0.0342)	0.1633 (0.0705)	14.0523 (20.3101)	< 0	< 0	0.0327 (0.1237)	< 0	0.1332 (0.044)	0.1119 (0.0345)	0.118 (0.0351)	0.1283 (0.0358)	0.1126 (0.0371)	0.1104 (0.0366)

*Using GWAS-MA summary statistics from the meta-analysis of ENIGMA and CHARGE cohorts. h2= SNP-based heritability. se= standard error. ICV= intracranial volume. A ratio < 0 usually indicates GC correction.

Extended data sheet (EDS) for results of posthoc analyses for pallidum and thalamus

Linkage disequilibrium score regression

EDS_Table 1. SNP heritability analyses for MRI brain volumes and genetic correlation with ADHD.

Brain region	N	Heritability	SE	Genetic correlation with ADHD	SE	Z	P
Pallidum	11,640	0.1557	0.0478	-0.04864	0.08893	-0.547	0.5844
Thalamus	11,694	0.095	0.0442	0.06506	0.1132	0.5747	0.5655

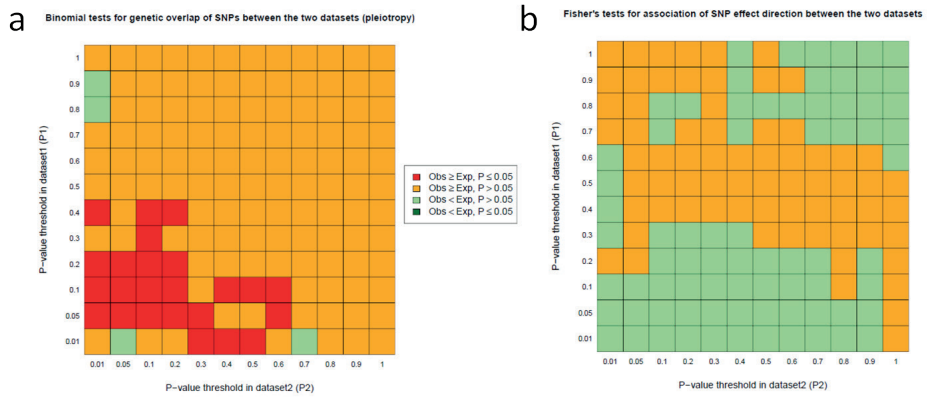
Heritability and genetic correlation were estimated by using free intercepts.

SNP effect concordance analysis

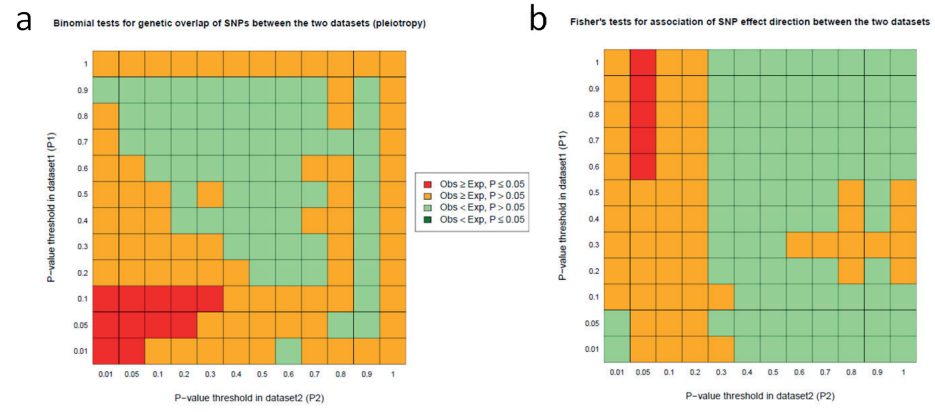
EDS_Table 2. Results of pleiotropy and concordance test of SNP Effect Concordance Analysis. Brain volume GWAS-MA was conditioned on ADHD GWAS-MA.

Brain volume	$P_{pleiotropy}$	$CI_{pleiotropy}$	$P_{concordance}$	$CI_{concordance}$	Direction of SNP effects
Pallidum	0.000999	5.12×10^{-5}-0.00564	1	0.996-1	/
Thalamus	0.00799	0.00406-0.0157	0.17	0.148-0.194	concordant

P-values and confidence intervals (CI) were obtained based on 1,000 permutations.



EDS_Figure 1: Global evidence of pleiotropy (**a**) and concordance (**b**) between ADHD GWAS and pallidum volume. P1 in the plot is the ADHD GWAS and P2 is the pallidum volume GWAS.



EDS_Figure 2: Global evidence of pleiotropy (**a**) and concordance (**b**) between ADHD GWAS and thalamus volume. P1 in the plot is the ADHD GWAS and P2 is the thalamus volume GWAS.

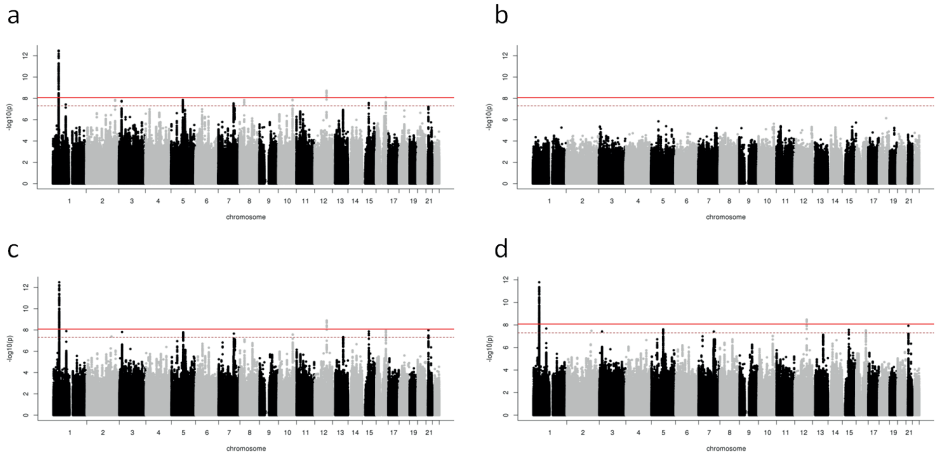
Sign tests

EDS_Table 3. Sign test results in brain volume cohorts.

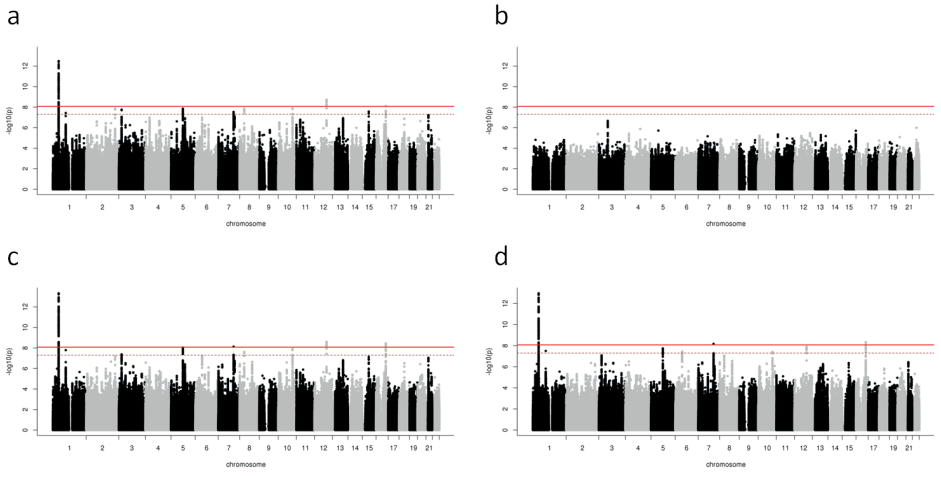
P threshold	Brain region	N opposite direction	Proportion	P
$< 5 \times 10^{-8}$	Pallidum	3	0.3	0.945
	Thalamus	3	0.3	0.945
$< 1 \times 10^{-6}$	Pallidum	16	0.46	0.75
	Thalamus	14	0.4	0.912
$< 1 \times 10^{-5}$	Pallidum	52	0.53	0.307
	Thalamus	43	0.44	0.906

Test of whether the proportion of index SNPs with estimated effects in the *opposite* direction as the ADHD GWAS-MA is greater than expected by chance. The expected proportion under the null hypothesis is 0.5. At threshold $P<5 \times 10^{-8}$ 4 index SNPs were not available in the brain volume GWAS-MA data, so were 9 at threshold $P<5 \times 10^{-6}$ and 34 at threshold $P<5 \times 10^{-5}$.

Weighted SNP meta-analyses



EDS_Figure 3: Common genetic variants associated with ADHD, pallidum volume and ADHD+pallidum volume. Shown here are Manhattan plots, in which every point represents a single genetic variant plotted according to its genomics position (x-axis) and its $-\log_{10}(P)$ for association with the respective trait (y-axis). The solid bright red line represents the study-wide genome-wide significance of $P<8.33x10^{-9}$, and the dashed dark red line represents the genome-wide significance of $P<5x10^{-8}$. (**a**) PGC+iPSYCH ADHD GWAS-MA. (**b**) ENIGMA pallidum volume GWAS-MA. (**c**) ADHD+pallidum volume weighted GWAS-MA. (**d**) ADHD+pallidum volume naive GWAS-MA.



EDS_Figure 4: Common genetic variants associated with ADHD, thalamus volume and ADHD+thalamus volume. Shown here are Manhattan plots, in which every point represents a single genetic variant plotted according to its genomics position (x-axis) and its $-\log_{10}(P)$ for association with the respective trait (y-axis). The solid bright red line represents the study-wide genome-wide significance of $P<8.33x10^{-9}$, and the dashed dark red line represents the genome-wide significance of $P<5x10^{-8}$. (**a**) PGC+iPSYCH ADHD GWAS-MA. (**b**) ENIGMA thalamus volume GWAS-MA. (**c**) ADHD+thalamus volume weighted GWAS-MA. (**d**) ADHD+thalamus volume naive GWAS-MA.

Gene-wide GWAS-MAs

EDS_Table 4: Results of MAGMA gene-based associations of all genes for ADHD and pallidum volume.

GENE	CHR	START	STOP	NSNPS	NPARAM	N	ZSTAT	P
6487	1	44073204	44496837	786	61	67014	7.3409	1.0612E-13
5792	1	43891708	44189343	521	60	67014	6.9833	1.4416E-12
23334	1	43755556	44019918	338	68	67014	6.7242	8.8282E-12
9682	1	44015797	44271189	533	43	67014	6.6261	1.7231E-11
1848	12	89641837	89846296	419	96	67014	6.5208	3.4966E-11
81888	1	43816674	44019938	272	57	67014	6.4185	6.8823E-11
4208	5	87914058	88299922	558	85	67014	6.4174	6.9333E-11
64834	1	43729068	43933745	266	53	67014	6.08	6.0103E-10
991	1	43724626	43928874	269	53	67014	6.0502	7.2337E-10
112950	1	43749579	43955483	247	67	67014	5.8561	2.3695E-09
4352	1	43703475	43920135	297	53	67014	5.7949	3.4171E-09
9048	1	44298992	44502912	365	60	67014	5.6449	8.2649E-09
80031	15	47376403	48166420	1966	166	67014	5.6188	9.6122E-09
9670	1	44312478	44533694	417	82	67014	5.5001	1.8984E-08
7075	1	43666566	43888781	372	91	67014	5.3069	5.5743E-08
22986	10	106300859	107124993	2208	143	67014	5.1518	1.2902E-07
389549	7	121841373	122051173	421	74	67014	5.1474	1.3207E-07
1802	1	44335653	44539043	396	72	67014	5.1283	1.4617E-07
533	1	44340118	44543972	408	77	67014	5.0018	2.8403E-07
149466	1	43647554	43851334	394	88	67014	4.9149	4.4418E-07
8704	1	44344874	44556843	428	94	67014	4.9063	4.6405E-07
93986	7	113626365	114433827	1141	150	67014	4.7381	1.0787E-06
282809	12	89713495	90020039	636	69	67014	4.6634	1.555E-06
151648	3	20102085	20327724	850	71	67014	4.6304	1.8244E-06
1006	16	61581169	62170939	1289	89	67014	4.6265	1.8597E-06
…	…	…	…	…	…	…	…	…

Genome-wide gene-based results of MAGMA analysis. Entrez-ID (GENE), Chromosome (CHR), Start (START) and end (STOP) position of the genes, number of SNPs in the genes (N SNPs), effective number of SNPs included (NPARAM), the total sample size (N), test statistics (ZSTAT), and gene-based p-values for ADHD GWAS-MA (tab 1), ENIGMA pallidum GWAS-MA (tab 2), and the weighted ADHD+pallidum GWAS-MA (tab 3) are shown. Genes were considered gene-wide significant, if they reached the Bonferroni correction threshold adjusted for the total number of genes (N=18,306; $P<2.731x10^{-6}$; genes marked in bold).

*The complete **EDS_Table 4** is available upon request.*

EDS_Table 5: Results of MAGMA gene-based associations of all genes for ADHD and thalamus volume.

GENE	CHR	START	STOP	NSNPS	NPARAM	N	ZSTAT	P
6487	1	44073204	44496837	786	61	67068	7.2671	1.84E-13
5792	1	43891708	44189343	521	60	67068	7.2529	2.04E-13
23334	1	43755556	44019918	338	68	67068	6.9625	1.67E-12
9682	1	44015797	44271189	533	43	67068	6.8068	4.99E-12
81888	1	43816674	44019938	272	57	67068	6.7098	9.74E-12
4208	5	87914058	88299922	558	85	67068	6.2764	1.73E-10
64834	1	43729068	43933745	266	53	67068	6.264	1.88E-10
991	1	43724626	43928874	269	53	67068	6.2428	2.15E-10
1848	12	89641837	89846296	419	96	67068	6.1181	4.73E-10
4352	1	43703475	43920135	297	53	67068	6.0989	5.34E-10
112950	1	43749579	43955483	247	67	67068	5.993	1.03E-09
80031	15	47376403	48166420	1966	166	67068	5.8007	3.30E-09
7075	1	43666566	43888781	372	91	67068	5.5799	1.20E-08
93986	7	1.14E+08	1.14E+08	1141	150	67068	5.5281	1.62E-08
22986	10	1.06E+08	1.07E+08	2208	143	67068	5.4915	1.99E-08
9048	1	44298992	44502912	365	60	67068	5.3491	4.42E-08
9670	1	44312478	44533694	417	82	67068	5.215	9.19E-08
149466	1	43647554	43851334	394	88	67068	5.1402	1.37E-07
1006	16	61581169	62170939	1289	89	67068	4.9275	4.17E-07
84966	1	18334240	18804977	1328	274	67068	4.895	4.92E-07
1802	1	44335653	44539043	396	72	67068	4.8937	4.95E-07
128218	1	43635665	43839673	436	84	67068	4.8762	5.41E-07
729852	7	7580342	8018854	1472	323	67068	4.8762	5.41E-07
389549	7	1.22E+08	1.22E+08	421	74	67068	4.8214	7.13E-07
533	1	44340118	44543972	408	77	67068	4.7676	9.32E-07
…	…	…	…	…	…	…	…	…

Genome-wide gene-based results of MAGMA analysis. Entrez-ID (GENE), Chromosome (CHR), Start (START) and end (STOP) position of the genes, number of SNPs in the genes (N SNPs), effective number of SNPs included (NPARAM), the total sample size (N), test statistics (ZSTAT), and gene-based p-values for ADHD GWAS-MA (tab 1), ENIGMA thalamus GWAS-MA (tab 2), and the weighted ADHD+ thalamus GWAS-MA (tab 3) are shown. Genes were considered gene-wide significant, if they reached the Bonferroni correction threshold adjusted for the total number of genes (N=18,306; $P<2.731x10^{-6}$; genes marked in bold).

*The complete **EDS_Table 5** is available upon request.*

EDS_Table 6: Comparison of genome-wide significant MAGMA gene-based results for ADHD and pallidum.

Gene Name	EntrezID	P_{ADHD}	$P_{pallidum}$	$P_{ADHD+pallidum}$
ST3GAL3	6487	5.57E-13	0.33343	1.06E-13
PTPRF	5792	7.6E-13	0.52848	1.44E-12
SZT2	23334	3.95E-12	0.79288	8.83E-12
KDM4A	9682	1.77E-11	0.40584	1.72E-11
DUSP6	1848	1.47E-11	0.58627	3.5E-11
HYI	81888	2.71E-11	0.7758	6.88E-11
MEF2C	4208	1.63E-10	0.2715	6.93E-11
ELOVL1	64834	3.55E-10	0.74887	6.01E-10
CDC20	991	4.11E-10	0.72683	7.23E-10
MED8	112950	1.7E-09	0.84361	2.37E-09
MPL	4352	1.31E-09	0.61228	3.42E-09
ARTN	9048	4.73E-08	0.29146	8.26E-09
SEMA6D	80031	1.62E-09	0.78929	9.61E-09
IPO13	9670	7.42E-08	0.19523	1.9E-08
TIE1	7075	1.7E-08	0.42991	5.57E-08
SORCS3	22986	2.22E-08	0.66127	1.29E-07
FEZF1	389549	9.51E-08	0.59552	1.32E-07
DPH2	1802	3.14E-07	0.20882	1.46E-07
ATP6V0B	533	6.26E-07	0.23988	2.84E-07
C1ORF210	149466	1.81E-07	0.29154	4.44E-07
B4GALT2	8704	1.03E-06	0.24352	4.64E-07
FOXP2	93986	4.14E-08	0.73244	1.08E-06
POC1B	282809	1.43E-06	0.72788	1.56E-06
CDH8	1006	4.54E-08	0.9676	1.86E-06
TMEM125	128218	6.17E-07	0.2747	1.89E-06
TALDO1	6888	2.54E-06	0.41757	2.16E-06

Genome-wide significant gene-based results of MAGMA for the 26 genes overlapping between the ADHD and ADHD+pallidum data sets. No gene showed stronger association (smaller cross-trait p-value and nominally significant p-value (P<0.05) in pallidum data set) in the cross-trait meta-analysis compared to the separate analyses of ADHD and pallidum.

EDS_Table 7: Comparison of genome-wide significant MAGMA gene-based results for ADHD and thalamus.

Gene Name	EntrezID	P_{ADHD}	$P_{thalamus}$	$P_{ADHD+thalamus}$
ST3GAL3	6487	2.76E-14	0.68469	1.84E-13
PTPRF	5792	1.23E-13	0.44022	2.04E-13
SZT2	23334	1.26E-12	0.46816	1.67E-12
KDM4A	9682	4.07E-12	0.38912	4.99E-12
HYI	81888	3.28E-12	0.37782	9.74E-12
MEF2C	4208	5.99E-10	0.41413	1.73E-10
ELOVL1	64834	1.68E-10	0.53903	1.88E-10
CDC20	991	1.52E-10	0.55619	2.15E-10
DUSP6	1848	4.53E-11	0.48078	4.73E-10
MPL	4352	1.84E-09	0.50396	5.34E-10
MED8	112950	2.39E-10	0.53891	1.03E-09
SEMA6D	80031	3.48E-09	0.57583	3.3E-09
TIE1	7075	1.11E-08	0.62267	1.2E-08
FOXP2	93986	3.5E-07	0.027619	**1.62E-08**
SORCS3	22986	1.64E-08	0.48316	1.99E-08
ARTN	9048	5.38E-08	0.89288	4.42E-08
IPO13	9670	3.79E-08	0.88159	9.19E-08
C1ORF210	149466	1.97E-07	0.79855	1.37E-07
CDH8	1006	4.93E-08	0.60852	4.17E-07
DPH2	1802	1.02E-07	0.91256	4.95E-07
TMEM125	128218	5.82E-07	0.79847	5.41E-07
FEZF1	389549	6E-07	0.76437	7.13E-07
ATP6V0B	533	2.43E-07	0.91095	9.32E-07
B4GALT2	8704	4.38E-07	0.9218	1.45E-06
ADD1	118	1.24E-06	0.69817	1.88E-06
KIZ	55857	6.47E-07	0.70382	2E-06

Genome-wide significant gene-based results of MAGMA for the 26 genes overlapping between the ADHD and ADHD+thalamus data sets. One gene showed stronger association (smaller cross-trait p-value and nominally significant p-value (P<0.05, marked in bold) in the thalamus data set) in the cross-trait meta-analysis compared to the separate analyses of ADHD and thalamus.

Reciprocal lookup of genome-wide significant associations

EDS_Table 8: Reciprocal look-up of significantly associated ADHD index SNPs in brain volume GWAS data

								Pallidum		Thalamus	
Index SNP	**A1**	**A2**	**chr**	**pos**	**OR_{ADHD}**	**P_{ADHD}**	**Proxy**	**LD to index (r2)**	**Zscore**	**P**	**Zscore**	**P**
rs11420276	G	GT	1	44184192	1.113	2.14×10^{-13}	rs112984125	0.976	-1.10924	0.2673	-1.53984	0.1236
rs1222063	A	G	1	96602440	1.101	3.07×10^{-8}	rs1222067	0.751	-0.25206	0.801	**0.066329**	0.9471
rs2391769	A	G	1	96978961	0.927	3.96×10^{-8}	/	/	**-1.44561**	0.1483	**-1.27605**	0.2019
rs9677504	A	G	2	215181889	1.124	1.39×10^{-8}	/	/	-2.12638	0.03347	-0.54654	0.5847
rs4858241	T	G	3	20669071	1.082	1.74×10^{-8}	/	/	-1.20436	0.2284	-0.40322	0.6868
rs28411770	T	C	4	31151456	1.09	1.15×10^{-8}	rs7674790	0.783	-1.80536	0.07101	-1.1548	0.2482
rs4916723	A	C	5	87854395	0.926	1.58×10^{-8}	/	/	**-0.70568**	0.4804	**-0.58023**	0.5618
rs304132	A	G	5	88215594	0.925	4.22×10^{-8}	/	/	**-0.21618**	0.8289	**-0.79279**	0.4279
rs5886709	G	GTC	7	114086133	1.079	1.66×10^{-8}	rs7795397	0.941	**0.465181**	0.6418	**0.601321**	0.5476
rs74760947	A	G	8	34352610	0.835	1.35×10^{-8}	/	/	0.287181	0.774	**-0.1583**	0.8742
rs11591402	A	T	10	106747354	0.911	1.34×10^{-8}	/	/	**-0.39996**	0.6892	**-0.66446**	0.5064
rs1427829	A	G	12	89760744	1.083	1.82×10^{-9}	/	/	**1.07631**	0.2818	**0.560579**	0.5751
rs281324	T	C	15	47754018	0.928	2.68×10^{-8}	/	/	**-1.21208**	0.2255	0.004724	0.9962
rs212178	A	G	16	72578131	0.891	7.68×10^{-9}	/	/	**-0.62581**	0.5314	**-1.25413**	0.2098

GWAS results from pallidum and thalamus brain volume GWASs for the genome-wide significant loci identified in the ADHD GWAS. Replication is tested for the index variant from the ADHD GWAS, or for a proxy variant when the index variant is not present in the brain volume cohorts. Effects (Z-score or odds ratio [OR] with reference to allele 1 [A1]) that are sign concordant with the ADHD GWAS are indicated in bold.

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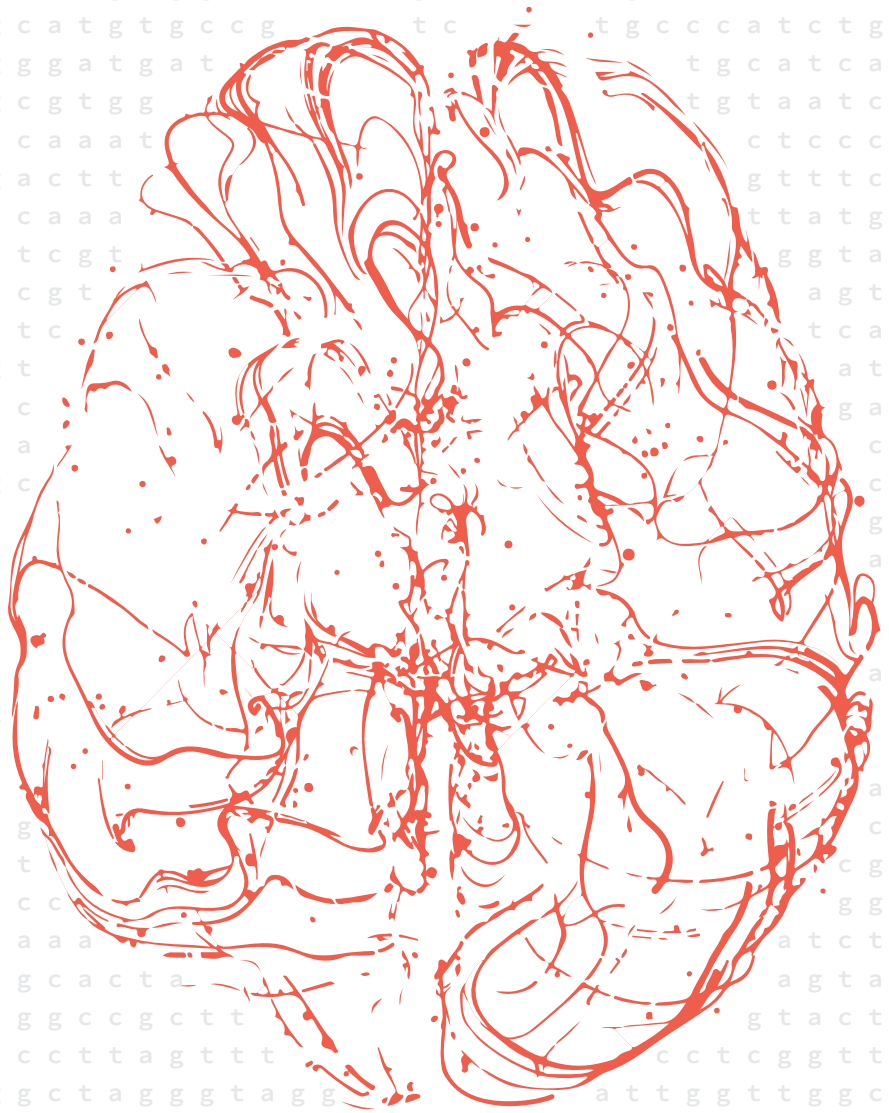

CHAPTER 11

Summary and general discussion

The work presented in this thesis focuses on identifying the genetic underpinnings of the highly heritable disorder ADHD and gaining insight into its underlying biological mechanisms by using different molecular genetics methodologies, brain imaging genetics approaches, and animal model systems. In this final chapter, the results from this thesis are summarized and discussed in the context of the existing literature.

Summary of approaches and results

Approaches for gene identification

Genetic factors play an important role in many neuropsychiatric disorders. Understanding which genetic variants are relevant for a particular disorder can yield insight into the pathophysiology and potentially (in the long run) lead to novel treatment options. GWA studies in ten-thousands of individuals have identified some genetic risk variants for ADHD, but the amount of variance in disease susceptibility that is explained by these variants is relatively small. The remaining, unexplained variance has been named 'missing heritability' and we aimed to uncover part of it in Part 1 of this thesis (**chapter 2** to **6**) by applying various approaches for the identification of ADHD risk variants.

We began with a candidate gene meta-analysis study in which we investigated the association of the ADHD-associated variable number tandem repeat (VNTR) polymorphism upstream of the *DRD5* gene with adult ADHD (**chapter 2**). We compiled data from six sites of the International Multicentre persistent ADHD CollaboraTion (IMpACT) and used the largest case-control sample investigated so far. We tested the association of the common *DRD5* alleles with categorically defined ADHD and with inattentive and hyperactive-impulsive symptom counts. Our findings provide evidence that, in contrast with earlier reports on childhood ADHD, none of the common *DRD5* alleles are associated with ADHD risk or ADHD symptom counts in adults.

Following the testing of a single candidate gene, we applied a hypothesis-generating approach and investigated common genetic variants (i.e., SNPs) on a genome-wide scale. Hypothesizing that genetic continuity exists between clinical ADHD and ADHD symptoms in the general population, in **chapter 3**, we report results of a genome-wide association meta-analysis of self-reported adult ADHD symptoms in adult population-based and case-only cohorts. The top-hit of the genome-wide meta-analysis was located within the *STXBP5-AS1* gene. This association was also observed in a meta-analysis of childhood ADHD symptom scores in eight population-based pediatric cohorts, validating the finding. In human HEK293 cells, expression of *STXBP5-AS1* enhanced the expression of a reporter-construct of *STXBP5*, known to be involved in SNARE complex formation. In mouse strains, *Stxbp5-AS1* transcript levels in the prefrontal cortex correlated with motor impulsivity. The results imply that the long non-coding RNA *STXBP5-AS1* is involved in ADHD symptom scores and point to vesicle transport as a biological mechanism involved in ADHD-related impulsivity levels.

It has been suggested that neurodevelopmental disorders, including ID, autism spectrum disorders (ASDs), ADHD, schizophrenia, and bipolar disorder, are seen as representing the diverse range of outcomes that follow from disrupted or deviant brain development (Owen and O'Donovan, 2017). This model is also known as the neurodevelopmental continuum and was based on emerging evidence for shared genetic and environmental risk factors, also predicting overlapping pathogenic mechanisms (reviewed in (Owen and O'Donovan, 2017)). Testing the concept of a neurodevelopmental continuum, we set out to investigate whether genes known to carry rare mutations in ID also contribute to ADHD risk through common variants. By this, we aimed to identify novel ADHD genes (**chapter 4**). A set of 396 ID genes was significantly associated with ADHD risk in two independent samples, and genetic variants in three genes, *MEF2C, TRAPPC9,* and *ST3GAL3*, were identified as consistent top-association findings across two different statistical methods. We then set out to functionally validate the newly identified ADHD candidate genes. In *Drosophila melanogaster*, two of the genes were conserved. Knockdown of *dMEF2* in monoaminergic neurons and knockdown of *dTRAPPC9* in circadian neurons showed increased locomotor activity and reduced sleep, implicating different biological pathways in ADHD etiology. The results of this study confirmed the genetic overlap of ADHD and ID, where genes, known to carry mutations in ID, confer ADHD risk through common variants. Utilizing this overlap, we identified, validated, and functionally characterized several novel ADHD genes.

An alternative to the use of large case-control cohorts or population-based samples for ADHD risk gene identification can be family-based approaches. In **chapter 5**, we used an innovative multi-step strategy to identify and validate a novel ADHD risk gene, combining the strength of a family study with the power provided by large-scale GWAS. In a single family, severely affected by ADHD and comorbid disorders, we applied microarray analysis to detect structural genetic variants co-segregating with ADHD. We identified a copy number gain in 8p23.3 containing three protein-coding genes (*ZNF596*, *FBXO25*, and *TDRP*). The identified novel ADHD candidate genes were tested - individually and as a set - for evidence of association with ADHD and other relevant psychiatric disorders based on common genetic variants. To functionally validate the identified ADHD risk genes, we characterized the effects of gene overexpression in *Drosophila melanogaster* using locomotor activity as functional read-out. Compared to the genetic background control, overexpression of the *FBXO25* orthologue lead to increased locomotor activity. Our findings suggest that *FBXO25* is a novel risk gene for ADHD.

In large pedigrees with multiple affected individuals, it had been hypothesized that rare (penetrant) mutations are likely to segregate with the phenotype of interest. Therefore, in **chapter 6**, we hypothesized that an innovative approach combining linkage analysis and whole-exome sequencing (WES) in multi-generation pedigrees containing multiple affected

individuals with exome-chip analysis in a large, independent cohort can point towards novel ADHD-related genes. From prioritized linkage regions, 24 genes harboring WES-identified rare variants were selected. Those genes were taken forward and were jointly analyzed in gene-set analyses using data of the independent exome-chip cohort of patients with persistent ADHD and healthy controls. Gene-wide analysis for the *AAED1* gene reached significance, and a single variant within the *AAED1* gene segregated with ADHD in one of the families. We concluded that the analysis strategy followed here is a fruitful approach for identifying novel ADHD candidate genes. Additionally, this study suggests that both rare and more frequent variants in multiple genes act together in contributing to ADHD risk even in individual multi-case families with an apparent dominant inheritance pattern.

Mapping mechanisms from gene to disorder

In the first part of this thesis we asked the question which molecular genetics mechanisms are underlying ADHD etiology. We identified individual genetic risk factors for ADHD and additionally, we obtained novel insights into the global genetic architecture of ADHD. The second part of the thesis focuses on different approaches that can help to map the biological mechanisms from gene to the disorder. Neurobiological parameters, such as brain structure, connectivity, and function, were used in humans and animal models to link genetic variation to ADHD symptomatology.

Our systematic review in **chapter 7** summarized the imaging genetics literature on three comorbid disorders (ID, ASD, and ADHD), focusing on studies of the effect of disease-linked genetic variants on brain structure and function. We aimed at identifying core brain mechanisms affected by disease-linked genetic factors, which are related to the individual disorders as well as to their clinical overlap. For ADHD and ASD, we selected replicated candidate genes implicated through common genetic variants. For ID, which is mainly caused by rare genetic variants, we included genes for relatively frequent forms of ID occurring comorbid with ADHD or ASD. Several findings were consistent across studies, implicating e.g. *SLC6A4/5HTT* in brain activation and functional connectivity related to emotion regulation. This review also enabled us to look at the overlap between studies in healthy individuals and those in patients (case-control designs). As it has been suggested from recent genome-wide studies investigating the genetics of brain structure as part of the ENIGMA Consortium, the genetic effects on brain structure are largely similar for healthy individuals and those with a psychiatric disorder (Hibar et al., 2015). Thus, brain imaging genetics studies with healthy participants can be very informative in discovering related brain correlates and in understanding the biological mechanisms leading to diseases of interest. Generally, many studies had small sample sizes, and hypothesis-based, brain region-specific studies were common. Therefore, replication of findings across studies was limited. Results from available studies confirm that imaging genetics can provide insight into the link

between genes, disease-related behavior, and the brain. However, the field is still in its early stages, and conclusions about mechanisms shared across disorders could not yet be drawn.

We then extended our work from the previous chapter and performed a systematic review of brain imaging genetics studies involving 62 ADHD candidate genes in childhood and adult ADHD cohorts (**chapter 8**). Almost exclusively, single genetic variants were studied, mostly focussing on dopamine-related genes. The conclusion from this review was that, while promising results have been reported, imaging genetics studies of ADHD are thus far hampered by methodological differences in study design and analysis methodology, as well as limited sample sizes. In this chapter, we also comprehensively discuss the need for complementary methods for the evaluation of the mechanisms underlying ADHD risk genes. We highlight the use of bioinformatic approaches that can help to integrate findings from different types of molecular studies. Additionally, the utilization of animal models is discussed, as these can provide proof of causality for genes and molecular processes found associated with ADHD. Moreover, we mention that psychiatric disorders can be modelled at the cellular level by using human induced pluripotent stem cell (hiPSC)- derived neurons. We conclude this chapter by emphasizing the importance of combining and integrating findings across levels for a better understanding of biological pathways from gene to disorder.

In **chapter 9**, we used an integrative approach with the aim to replicate the earlier reported association of *GIT1* with ADHD and to investigate its role in cognitive and brain phenotypes. Association of *GIT1* with ADHD or any related measures, such as sustained attention, working memory, and brain volume measures was not confirmed in much larger sample sizes than used in the primary study. We did find a functional genetic variant to be an expression quantitative trait locus (eQTL) for *GIT1*. Moreover, *Git* knockdown in *Drosophila* caused abnormal synapse and dendrite morphology, but did not affect behavior (i.e. locomotor activity). Our results indicate that despite GIT1's regulation of neuronal morphology, alterations in gene expression do not appear to have ADHD-related behavioral consequences and do not appear to alter brain volume in humans.

ADHD is a common and highly heritable neurodevelopmental disorder with a complex pathophysiology, where genetic risk is hypothesized to be mediated by alterations in structure and function of diverse brain networks. In **chapter 10**, we tested one aspect of this hypothesis by investigating the genetic overlap of ADHD with subcortical brain volumes and intracranial volume (ICV). At the level of common variant genetic architecture, we discovered a significant negative genetic correlation between ADHD and ICV. Meta-analysis of individual variants found significant loci associated with both ADHD risk and ICV; additional loci were identified for ADHD and amygdala, caudate nucleus, and putamen volumes. Gene-set analysis in the ADHD-ICV meta-analytic data showed significant association with variation in neurite outgrowth-related genes. This is the first study to show subtle but significant global and single variant level genetic correlations derived from polygenic overlap between ADHD and brain volumes. The results generate new hypotheses about neurobiological

mechanisms involved in ADHD etiology, the rather modest overlap reinforces the need to consider other structural and functional brain metrics and explore the role of environmental factors.

Interpretation in the context of existing literature

Approaches for gene identification

The first part of this thesis describes a set of established and novel molecular genetics approaches that have been used in order to gain more insight into the genetic factors underlying ADHD etiology. ADHD is a polygenic and multifactorial disorder, i.e., both genetic factors as well as environmental factors are involved (Faraone et al., 2015; Thapar and Cooper, 2016). As schematically depicted in **Figure 1**, the cumulative (but individually weak) effect of common genetic variants contribute to ADHD susceptibility in an individual. Recently, the SNP-based heritability of ADHD was estimated to be approximately 22% (Demontis et al., 2017), i.e., this is the proportion of phenotypic variance attributable to common genetic variants (SNPs). This is much lower than the heritability estimated from twin studies (70-80% (Faraone et al., 2005; Larsson et al., 2013b)), suggesting that other types of genetic variation (and genetic models) contribute to ADHD etiology as well. The resulting composition of the genetic architecture, with its set of common genetic variants, interacting between them and combined with potentially intermediate to large effects of rare genetic variation, is probably unique for each affected individual (**Figure 1**). The next section relates the results of the different chapters to the existing literature and addresses the crucial question of "*What have we learned about the genetic architecture of ADHD?*" by describing four main emerging facets.

I. The choice of study design: candidate gene studies versus genome-wide approaches

The search for genetic variants contributing to ADHD risk was initially based on the hypothesis that a common disorder, such as ADHD, will most likely be caused by common genetic variants. Early studies, starting well before the publication of the human genome sequence, focused on investigating single candidate genes for ADHD, of which most were related to catecholaminergic functions, such as dopamine, noradrenalin, and serotonin neurotransmission. Meta-analyses of candidate gene studies have been helpful in more robustly estimating the strength of association between such single genetic variants and ADHD. In this thesis, **chapter 2** and **chapter 9** investigated a single gene in a candidate gene approach. Both the investigated *DRD5* (**chapter 2**) and *GIT1* (**chapter 9**) gene had been associated with ADHD before (Won et al., 2011; Wu et al., 2012). However, the initial studies had a limited samples size and were therefore not reasonably powered. Moreover, the previous studies were either performed in a childhood ADHD sample only (*DRD5*, **chapter 2**) or in a population with a different ethnicity (*GIT1*, **chapter 9**). Thus, our (negative) association results add important information to the literature, as the study presented in **chapter 2** is the first performing an association analysis of the *DRD5* VNTR in patients with persistent ADHD. Additionally, we investigated the association of *GIT1* and ADHD risk in a sample of European decent (**chapter 9**). Overall, these two studies show the need and the added value of replication studies and meta-analysis.

Although some promising candidate genes have been identified previously, none of the historical ADHD candidate genes are among the strongest associated genes in the recent PGC+iPSYCH ADHD GWAS meta-analysis (Demontis et al., 2017). Similar findings for schizophrenia also indicate that the most investigated candidate gene hypotheses of schizophrenia (with the exception of those for *DRD2*) are not well supported by GWASs (Johnson et al., 2017). In the field of genetics, candidate-based single gene analyses have largely fallen out of favor owing to concerns about low power, false positives, low replication rates (Ioannidis et al., 2011; Koenen et al., 2013), and insufficient biological knowledge to correctly identify plausible candidate genes. Given the increasingly low cost of whole-genome array data, genome-wide hypothesis-generating analyses in sufficiently powered cohorts are becoming available for many complex traits. As single variant association analyses of ADHD diagnostic status have shown considerable pitfalls (Neale et al., 2010; Thapar et al., 2013), this thesis describes various more suitable methods to investigate genetic associations in ADHD. The studies presented in **chapter 5** and **6** combined analysis strategies to identify both common and rare variants associated with ADHD risk. Whenever applicable, we aimed to maximize statistical power by applying gene-based or gene-set association analyses (**chapter 3-6**, **9**, and **10**). This methodological approach allows the investigation of a combined effect of common genetic variants. Genome-wide, hypothesis-generating approaches were applied in the studies described in **chapter 3** and **10**.

II. Aspects of neurodevelopmental continua in ADHD

A continuum – phenotypic and genetic – from population traits to clinical disorder

ADHD is characterized by age-inappropriate, sustained symptoms of inattention and hyperactivity/impulsivity (American Psychiatric Association, 2013). Earlier studies showed that polygenic risk for ADHD is associated with ADHD symptoms in the population (Martin et al., 2014) (albeit with a negligible proportion of variance explained), confirming the conclusion from twin studies that genes contributing to ADHD risk also regulate the expression of non-clinical levels of ADHD symptoms. This suggestion that etiological influences on ADHD symptoms are distributed throughout the population, is consistent with a liability model (Caspi et al., 2008). The concept of the *phenotypic continuum* and the liability model are shown in **Figure 1**. The liability-threshold model posits that the phenotypic outcome can be determined quantitatively by the combined effects of genetic

load and environmental factors. If cumulative predisposition exceeds a certain threshold value, individuals manifest with the clinical disorder, such as ADHD.

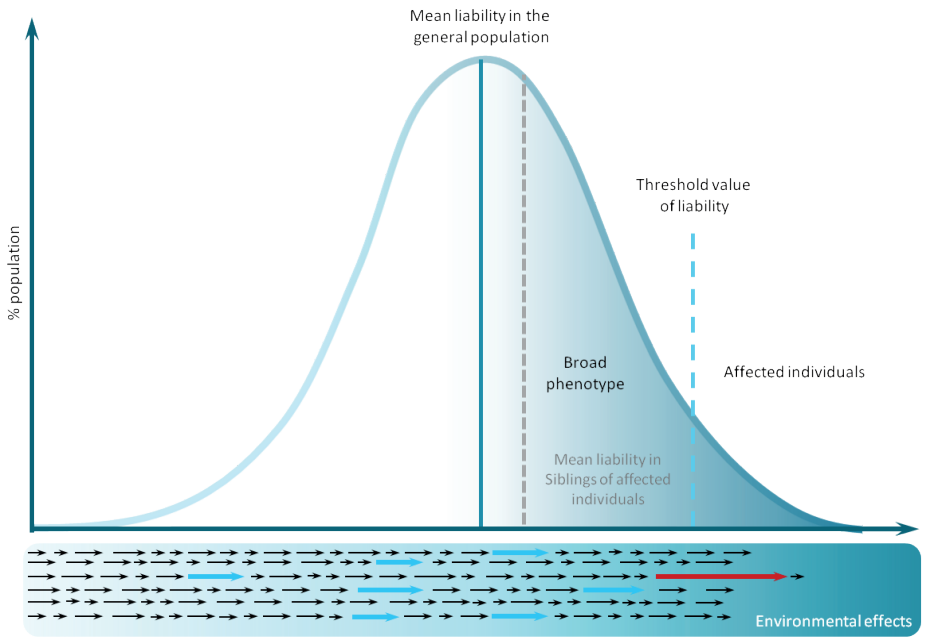

Figure 1. The model of ADHD liability. The liability-threshold model, assuming a Gaussian distribution of a continuous liability in the general population, posits phenotypic outcome can be determined quantitatively by the combined effects of genetic load and environmental factors. If cumulative predisposition exceeds a certain threshold value, individuals manifest with the clinical syndrome (continuum). Weak effects of common genetic variants (black arrows), intermediate and potentially larger effects of rare variation in some individuals (blue and red arrows, respectively), and any environmental effects (lower bar) affect liability. Each line of arrows represents an affected individual. The figure was adapted from (Chaste et al., 2017).

In line with those findings, we tested the association of the common *DRD5* alleles with categorically defined ADHD and with inattentive and hyperactive-impulsive symptom counts in **chapter 2**. It is of interest to assess which symptom domain the genetic variants are associated with. However, this study was performed on a small scale, since only a single gene was investigated. The characteristic domains of inattention and hyperactivity/impulsivity are separate domains of ADHD's psychopathology, with a genetic correlation of around 0.6 (Larsson et al., 2013a). This reflects a substantial genetic overlap but also implies that domain-specific genetic influences exist (Bidwell et al., 2017), supporting a dimensional approach to genetic studies of ADHD.

A critical question in study design is, which study population to use? The study of patients may be obvious, but is the effect of 'disease/disorder genes' really restricted to patients? In addition to the work discussed above, recent findings show that most of the (identified)

risk variants are common in the general population, with minor allele frequencies between 1-50% (Gormley et al., 2016; Schizophrenia Working Group of the Psychiatric Genomics et al., 2014). Is it therefore possible that common risk variants only cause the disorder in a subset of carriers, e.g., because they exert an effect only in combination with specific other risk factors? This is not the most likely situation, and an alternative explanation can be that patients with a clinical diagnosis of ADHD are at the extreme end of a continuous spectrum, with non-diseased carriers of the risk variants showing less severe phenotypes. Such a continuity of ADHD (disorder and symptoms) can also be seen on the genetic level (*genetic continuum*). Genetic risk scores for clinical ADHD were associated with ADHD symptom levels in the general population (Groen-Blokhuis et al., 2014; Martin et al., 2014) and symptom trajectories (Riglin et al., 2016), suggesting a genetic overlap between clinical ADHD (state) and increasing symptoms (trait). Additional evidence for this is provided by a recent study that investigated the genetic correlation between a clinical (mainly childhood) ADHD sample and ADHD symptoms in the general population (again mainly childhood cohorts) (Demontis et al., 2017; Middeldorp et al., 2016). The genetic correlation between both phenotypes was very high (r_g=0.943, SE=0.204), suggesting a strong genetic overlap between the disorder and the trait (Demontis et al., 2017).

Most of the studies in this thesis used clinical populations and a case-control design, but in the study in **chapter 3** we used the opportunities provided by studying (mainly) general population cohorts. Inattention and hyperactivity/impulsivity symptoms can be reliably assessed in population-based cohorts based on rating scales (Larsson et al., 2013a). This opens the opportunity to collect large population-based samples to identify disorder-relevant genetic variants. In **chapter 3** we used the power of population studies of ADHD symptoms in adults by applying a genome-wide hypothesis-generating approach. The results of our study show that self-reported adult ADHD symptoms measured in the general population have a genetic component, and that performing population-based GWAS meta-analysis of adult ADHD symptoms can provide novel insights into the genetic underpinnings of ADHD symptoms. However, we did not find any genome-wide significant association, potentially indicating that the size of our population-based samples was still not large enough. Moreover, we had to include the combination of three different phenotyping instruments (i.e., ADHD symptoms rating scales), which may have increased the heterogeneity and therefore may have lowered the overall power. To our surprise, we did not observe significant genetic correlation with clinical ADHD, which may be due to the limitations described above. Future studies will prove if simply expanding sample size is indeed a winning strategy for driving discovery of adult ADHD symptoms in the general population. Generally, the numbers needed will depend on the phenotype's heritability and polygenicity and the effect sizes of the contributing SNPs (Holland et al., 2016).

The concept of the *genetic continuum* is not only limited to ADHD symptoms, but can also be expanded to other ADHD-related traits. Also trans-diagnostic effects have been

observed. In the case of schizophrenia, the most compelling evidence began to emerge about ten years ago, particularly from genomic studies that implicated large and rare CNVs in conferring risk to schizophrenia and showed that the same variants also confer risk to intellectual disability, autism spectrum disorders, and ADHD (Kirov et al., 2014; Rutter et al., 2006). This model is based on emerging evidence for sharing of genetic and environmental risk factors and predicts that there are also likely to be overlapping pathogenic mechanisms (Owen and O'Donovan, 2017). For ADHD, studies showed that the genetic contribution (of common genetic variants) to ADHD overlaps with that of other psychiatric (and non-psychiatric) disorders (Anttila et al., 2017; Demontis et al., 2017). In line with this idea, the cross-phenotype effects can be investigated in other ADHD-related traits, e.g. brain volume measures, as was studied in **chapter 10**. There, we showed that genetic overlap between genes for ADHD risk and ICV is significant, but overall the genetic correlation with brain volume measures is limited.

A mutational continuum in neurodevelopmental disorders

Another concept emerging from this thesis is that of a *mutational continuum*: this concept suggests that genetic mechanisms operate across a range of disorders (Owen, 2012; Owen and O'Donovan, 2017), i.e., the same genes are involved in different (neurodevelopmental) disorders, where the severity of genetic defects may determine the specific disorder expressed by a patient (**Figure 2**). In this mutational continuum, neurodevelopmental disorders, including e.g. intellectual disability, autism spectrum disorder, schizophrenia, and ADHD, are seen as representing the diverse range of outcomes that follow from disrupted or deviant brain development. These findings of shared genetic risk and shared pathogenic mechanisms among neurodevelopmental disorders also support the view that these disorders lie on a gradient of severity, implying that they overlap to some extent quantitatively as well as qualitatively (**Figure 2**) (Owen and O'Donovan, 2017). Since many of the individual genetic association are shared across multiple psychiatric disorders, this points to extensive biological pleiotropy (O'Donovan and Owen, 2016). Findings from **chapter 4** add to the concept of the mutational continuum, as it demonstrates the genetic continuity of ADHD and ID, where genes, known to carry mutations in ID, contribute to ADHD risk through common variants. There are also examples for the mutational continuum in other (types of) phenotypes. For example, deletions in the gene *AKT3* cause microcephaly syndromes (Boland et al., 2007), and *AKT3* duplications cause macrocephaly (Wang et al., 2013). This gene is part of brain growth pathways, which were recently found to also be strongly linked to ICV in the general population (Adams et al., 2016b; Reijnders et al., 2017). Moreover, heterogeneity and incomplete penetrance suggest that the classical distinction between Mendelian and complex diseases is not always absolute and that a continuum may exist between purely Mendelian diseases and most complex diseases. Additionally, a more recent study showed that genes harboring both causal variants for Mendelian disorders

and risk factors for complex disease traits, tend to present higher functional relevance in protein networks and show higher expression levels than genes associated only with complex disorders (Spataro et al., 2017).

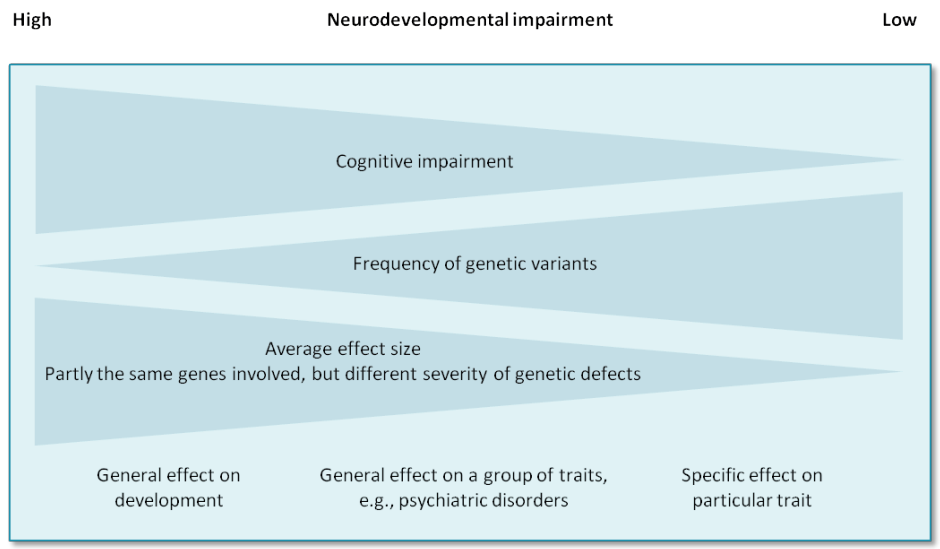

Figure 2. The mutational continuum in neurodevelopmental disorders. The relative impact of genetic variants is shown, ranging from damaging point mutations and CNVs to common variants. Damaging variants are less frequent in the population compared to common genetic variants which are less penetrant. If the cognitive impairment is high, e.g. in ID, rare damaging mutations with a general effect on neurodevelopment are thought to be causal. In the case of ADHD, the cognitive impairment is less severe and the genetic contribution to the disorder is mainly coming from common genetic variants with smaller effects. The mutational continuum suggests that genetic mechanisms operate across a range of disorders, i.e., the same genes are involved in different (neurodevelopmental) disorders. In some instances, an increasing severity of genetic effects is observed (e.g., genes affected by rare genetic variation in ID patients also contribute to multifactorial ADHD risk through common genetic variation, **chapter 4**).

III. The role of rare variants in ADHD

Most genetics research in ADHD focused on common genetic variants, mainly motivated by the common disorder – common variant theory. Based on the results of GWASs, we know that the amount of variance in disease susceptibility explained by these common genetic variants is small. It was expected that rare genetic variants have stronger effects on ADHD risk (see introduction **Figure 1**), and that, therefore, genetic studies focusing on the less frequent spectrum of genetic variation could help unravel part of the so-called 'missing heritability'. For several other psychiatric disorders, it is known that rare variants have a role in the disorder's etiology. In the case of schizophrenia, not only large, rare structural genetic variants (CNVs), but also rare single nucleotide variants (SNVs) contribute to disease risk, as increased frequencies of *de novo* point mutations have been observed in samples of individuals with schizophrenia in comparison to control individuals (Fromer et al., 2014).

WES studies have been successful in identifying rare risk alleles for neurodevelopmental/ psychiatric disorders, such as autism spectrum disorders (Iossifov et al., 2014) and schizophrenia (Purcell et al., 2014).

Different designs can be used for studies aiming at identifying rare variants linked to complex disorders. Most comparable to genomic studies of common genetic variants is the approach to collect large case-control data sets. For ADHD, a small number of studies followed this strategy yet. Initial WES work revealed an enrichment of rare variants in a gene-set of previously defined candidate genes for ADHD (Demontis et al., 2016). Additionally, a more hypothesis-generating approach was followed by performing a genome-wide analysis based on exome-chip data (Zayats et al., 2016). However, both studies are of limited sample size (n_{WES}=205 and $n_{exome\text{-}chip}$=9,365), and larger samples are needed for robust case-control WES studies (e.g., as for schizophrenia (Purcell et al., 2014)) to clarify the range of effect sizes of these rare variants. Alternative approaches come from integrative bioinformatic analyses. A recent study ranked genes, found in rare CNVs associated with ADHD, according to prior biological knowledge obtained from both human and cross-species databases. By this, novel ADHD candidate genes were prioritized (Harich et al., submitted).

In a second approach, large pedigrees with multiple affected individuals have been subjected to genetics research, as it had been hypothesized that rare (and penetrant) mutations would segregate with the phenotype of interest. In two chapters of this thesis (**chapter 5** and **chapter 6**), we hypothesized that families with multiple affected family members are enriched in rare genetic risk factors for ADHD and thus we would be able to limit genetic heterogeneity, since rare variants with potentially higher penetrance may cause the disorders in these ADHD families. Therefore, we employed the extended pedigree-based approach, in which one screens for segregation of rare variants with disease across multiple affected individuals. Importantly, the results from both chapters imply that - despite the apparent dominant segregation pattern - ADHD is not a monogenic disorder in the pedigrees investigated. The observed (imperfect) segregation pattern is similar to findings in previous linkage studies of ADHD (Lesch et al., 2008; Zhou et al., 2008) and other neurodevelopmental disorders (Chapman et al., 2015). While rare variants were observed in the individual families, common genetic variants in the same genes are associated with ADHD in larger case-control cohorts. Thus, for all families presented in **chapter 5** and **chapter 6**, we may have to assume a more complex combination of rare and common variants contributing to the ADHD phenotype. WES and WGS studies in multiplex autism families (Shi et al., 2013; Toma et al., 2014) and familial bipolar disorder (Goes et al., 2016) provide some evidence for rare, inherited variants segregating with the disorder. However, for ADHD, Mendelian (monogenic) forms may not exist (in the absence of comorbid ID), even not in familial ADHD pedigrees. Interestingly, families ascertained for having two or more children with autism spectrum disorders and simplex families showed distinct patterns of

genetic risk: the rate of large, rare *de novo* CNVs is lower in multiplex families, and there is an increased burden of large, rare inherited CNVs (Leppa et al., 2016).

A third approach, which has successfully been applied in other neurodevelopmental disorders, especially in autism spectrum disorders (Hashimoto et al., 2016; Iossifov et al., 2014; O'Roak et al., 2011; O'Roak et al., 2012), is a trio-approach with sporadic patients and their parents. In this approach, it is assumed that the occurrence of the disorder in the patient is due to *de novo* mutations. However, in ADHD this approach may seem less promising, since the disorder does not reduce reproductive fitness as it does e.g., in autism spectrum disorders. Therefore, sporadic ADHD cases are less frequent, and familial aggregation of ADHD is more frequently observed (Chen et al., 2017a). On the other hand, recent evidence from the Swedish population registries suggests that ADHD risk is strongly increased in the offspring of older fathers (<45 years) (D'Onofrio et al., 2014), which may be linked to an age-dependent increase in mutation rate in the paternal germline (Kong et al., 2012). Moreover, it had been suggested that the cognitive profile of families with only one affected child differs from that of families with multiple affected children (Oerlemans et al., 2015a; Oerlemans et al., 2015b). This may indicate that the trio-design could be promising in ADHD after all. Indeed, an initial study suggests that WES of sporadic ADHD can identify novel and potentially pathogenic *de novo* variants (Kim et al., 2017a).

Overall, rare variants contribute to the genetic architecture of ADHD, however, the exact contribution and impact of such rare variants on ADHD etiology still has to be estimated. Similarly, the role of rare variants in the onset and persistence of ADHD awaits further studies. Many of the individual genetic associations for other types of genetic variation, such as CNVs and SNPs, are shared across multiple neurodevelopmental disorders in a way that points to extensive biological pleiotropy. Thus, the specificity of rare SNVs for ADHD should also be assessed.

IV. Changes in the genetic contribution across the lifespan

Although the majority of children with ADHD will not continue to meet the full set of criteria for ADHD as adults, the persistence of either functional impairment (Faraone et al., 2006) or subthreshold impairing symptoms into adulthood is high (Biederman et al., 2000). Results of prospective longitudinal studies suggest that approximately two-thirds of youth with ADHD retain impairing symptoms of the disorder in adulthood (Faraone et al., 2006). However, genetic and environmental links between ADHD in childhood and the adult manifestation of the disorder is poorly understood, mainly because of a lack of longitudinal studies. A Swedish longitudinal twin study showed that one-fourth of the heritability in adulthood was shared with childhood manifestations of attention problems, indicating the presence of stable genetic risk factors that influence ADHD symptoms over time; the study also revealed evidence of new genetic factors that emerged during the transition from child through adolescent to young adult development (Chang et al., 2013), suggesting

that ADHD is a developmentally complex phenotype characterized by both stable and shifting genetic influences across the lifespan (Biederman et al., 2010; Faraone et al., 2006). This means that only part of the factors that contribute to ADHD onset also contribute to persistence of ADHD. Since most studies used twin data to assess the proportional impact of genes and the environment on interindividual differences in the developmental course of ADHD symptom domains (Kuntsi et al., 2005; Pingault et al., 2015; Rietveld et al., 2004), the contribution of individual genetic factors has not yet been studied. The results of our candidate gene association study in **chapter 2** contribute evidence that the risk alleles contributing to ADHD symptoms partly differ by age. While evidence for an association of *DRD5* VNTR alleles with ADHD in children had been strengthened by meta-analysis (Wu et al., 2012), we did not find a similar effect in a meta-analysis of persistent ADHD in adults. Differential association of genetic variants with ADHD observed in childhood and adulthood has also been reported for the dopamine transporter gene (*DAT1/SLC6A3*) (Franke et al., 2010). A potential underlying mechanism for the *DAT1* gene has been suggested by positron emission tomography (PET) studies of the Volkow group showing that genotypes are linked to different developmental trajectories of DAT availability (Volkow et al., 1996) (see also (Shumay et al., 2011)).

On a genome-wide scale, a high genetic correlation between clinical ADHD in children and persistent ADHD in adults has recently been observed based on common genetic variants (~81%; (Ribases et al., 2017)). However, the results need to be interpreted cautiously, since methodological explanations for this phenomenon cannot yet be ruled out. This finding could also indicate that the difference between childhood and persistent ADHD is coming from rare (or other structural) genetic variants. Moreover, the findings could be related to epigenetic effects, and/or gene-gene and gene-environment interactions.

Mapping mechanisms from gene to disorder

With the recent advances in genomics by both GWASs (i.e. larger and more powerful studies yielding many significant associations, and providing robust candidate genes for follow-up studies) and next-generation sequencing (WGS and WES allow evaluation of the genome in much greater depth than before), more and more genetic variants are being identified that potentially contribute to ADHD etiology. This implies that the field of psychiatric genetics is now shifting focus from "simply" gene-finding to the characterization of the underlying biological mechanisms. So the critical question, that the following section is about to answer, is "*How to turn information into knowledge*?" and to stress potential strategies to study the relevance of genetic variants. For this, multi-level research approaches are indispensible as they can shed light on multiple levels of complexity and can help to identify the mechanisms underlying the effects of ADHD genes on behavior and disease (**Figure 3**).

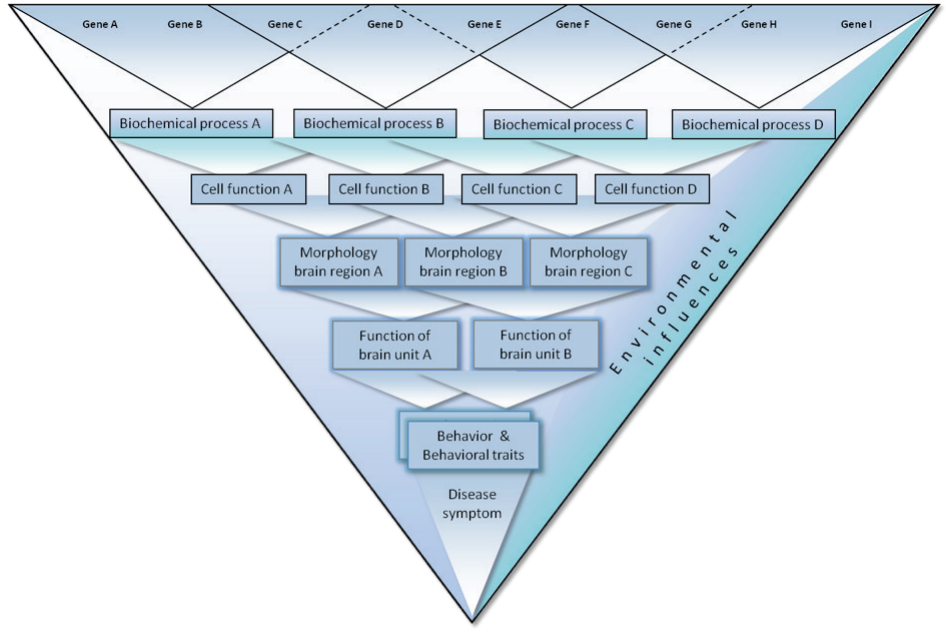

Figure 3. This schematic representation of pathways from gene to disease at different levels of complexity in psychiatric genetics. The figure shows the convergence of the effects of multiple genetic and environmental factors in disease symptoms. It has been modified from a previous publication (Figure 1, (Franke et al., 2009)). Polygenicity (schematically depicted by gene A to I (top line)) is suggested to be involved in causing disease symptoms. Groups of genes converge on different biochemical pathways and biological processes contributing to disease. These can be studied at various biological levels, e.g. biochemical processes and cell function can be assessed by biological assays in cell or animal models, by measuring e.g. neuron morphology or synaptic functioning. Bioinformatic pathway and network analyses can help to integrate data from various sources and to identify molecular networks or cellular processes in which ADHD-related genes are enriched. Neuroimaging methods (structural and functional) can be applied to assess relevant phenotypes at the level of brain morphology ('Morphology brain region A-C'). 'Function of brain units', can be e.g. investigated by functional MRI or through performance measurements on neuropsychological tests. Aberrations at this level can result in altered behavior and disease-related behavioral traits, that subsequently lead to disease symptoms. Environmental influences can impact all levels, and need more attention in future studies. The figure was adapted from (Klein et al., 2017).

V. Bioinformatics approaches integrate findings of molecular studies

The step from a GWAS-associated locus to identifying the casual variation underlying the association can be challenging. In this, bioinformatics approaches can be helpful as they can follow-up on genetic discoveries and serve to integrate findings from different types of molecular studies. Bioinformatics is a broad field of research, and although it is not the focus of this thesis, results from bioinformatic approaches impacted on multiple chapters in this thesis. Bioinformatic analyses can help in the clarification of the actual effects of risk variants on gene expression and regulation; as disease risk variants are often found in non-protein coding sequences, their molecular consequences are difficult to evaluate (Civelek and Lusis, 2014; Paul et al., 2014). Moreover, bioinformatics can help to unravel the molecular networks

and cellular processes that an ADHD risk gene is involved in; genetic factors associated with ADHD are distributed across the genome, but they have been found to be enriched within functional processes. Identification of these clustered functional networks can help to understand the biological processes underlying ADHD etiology. For example, an early study from our lab showed that ADHD GWASs association signals converged on the biological process of directed neurite outgrowth (Poelmans et al., 2011). This result has been taken forward in the study described in **chapter 10**. More recent enrichment analyses revealed that ADHD GWAS association signals were most enriched in biological functions related to nervous system development, neuron projection morphogenesis, cell-cell communication, glutamatergic synapse/receptor signalling, and multicellular organismal development (Hawi et al., 2015; Mooney et al., 2016; Yang et al., 2013).

VI. Modeling disease in animal models

In order to fully understand the role of ADHD risk genes in brain structure, cognition, and behavior, it is essential to manipulate genes and to monitor effects of such manipulation on molecular and cellular processes, as well as on the behavioral level. Of course, the animal models presented in this thesis should not be viewed as an attempt to model human ADHD in its entire complexity. However, the investigation of specific aspects of disorders has strongly advanced out understanding of disorder's pathologies (Gatto and Broadie, 2011). Generally, molecular and cellular processes are well conserved between species. Moreover, specific ADHD-related behavioral traits have been shown to be suited for being modeled in animal models, since these are relatively basic processes, that are common in most species, such as locomotion. Animal models were used in this thesis in multiple chapters. In **chapter 3**, mouse strains featuring different levels of impulsivity were investigated. *Stxbp-AS1* transcript levels in the prefrontal cortex strongly correlated with motor impulsivity levels as measured in a reaction time task in different mouse strains. This validation provided additional evidence that *STXBP5-AS1* is involved in ADHD-related impulsivity levels. A more unconventional animal model for ADHD used in our studies was the fruit fly (*Drosophila melanogaster*) (van der Voet et al., 2016; van der Voet et al., 2014). This model has several advantages over classical (murine) models, such as being relatively inexpensive, having a wide range of genomic tools available that allow spatial and temporal manipulation, and being highly suitable for fast studies of (candidate) genes. *Drosophila melanogaster* seems to be a suitable model for ADHD research, as it has been shown that flies exhibited increased locomotor activity, when expression of ADHD candidate genes was altered, and ADHD medication was able to reverse the behavioral phenotype (van der Voet et al., 2016). Among the behaviors that can be reliably assessed in *Drosophila* are locomotor activity and sleep, which are known to vary in a well-regulated day/night rhythm. *Drosophila melanogaster* was used in **chapter 4** and **5** to investigate the effects of altered expression levels of the newly identified ADHD candidate genes. Most importantly, the results of **chapter 4** and **5**

show increased locomotor activity and reduced sleep in the mutant fly lines. This provided evidence that specific and isolated manipulation of the genes resulting in altered expression can lead to ADHD-related phenotypes. Interestingly, for all three genes investigated, the hyperactivity was seen primarily during the dark period (night). For both the *TRAPPC9* gene (**chapter 4**) and the *FBXO25* gene (**chapter 5**) these are the first reports providing evidence for those gene having a role in the nervous system. A similar pattern has been observed in a previous study of our group, which showed that manipulation of ADHD-associated genes in *Drosophila* yields an ADHD-relevant, specific, and readily recognizable locomotor phenotype that is indicative of dysregulated signaling in a dopaminergic circuit (van der Voet et al., 2016). Importantly, in **chapter 4** we dissected the role of two neuronal substrates can help to reveal the neurotransmitter signaling pathways that contribute to ADHD. We investigated both the dopaminergic and circadian components, since both circuits have a well-established role in ADHD (Baird et al., 2012; Gowrishankar et al., 2014). Studying neuronal subtypes is important to reveal phenotypes that might otherwise be masked: earlier studies have identified two opposing circuits within the part of *Drosophila* brain that regulates learning, memory, activity, and sleep (Sitaraman et al., 2015). Our results reveal an increased night activity signature of the knockdown of *dMEF2* that indicated the importance of a dopaminergic signaling dysregulation in ADHD-relevant behavior. Additionally, knockdown of *dTRAPPC9* in the circadian rhythm circuit showed ADHD-related behavior with nightly increased activity and reduced sleep. This means that manipulation of ADHD-associated genes can affect locomotion in a cell type specific manner. However, future research is needed to understand the underlying molecular mechanisms and identify proteins regulated by the newly identified ADHD candidate genes, probably even in distinct neuronal populations. This will allow to better understand the etiology of the aberrant behaviour caused by variation in these genes and potentially also allows individualization of treatment at a later stage.

The second purpose of using *Drosophila melanogaster* as a model system in this thesis was to functionally *characterize* novel ADHD genes. Gene function is studied in detail on multiple levels to determine its relevance for the disorder. This was done in **chapter 9**, in which the effects of *Git* knockdown on behavior (locomotor activity) and neuronal connectivity on a cellular level (dendritic branching, axon guidance, synapse development) are described. Our observations support an important role for *Git* in synaptic and dendritic organization, as neuronal *Git* RNAi knockdown interferes with synaptic terminal branching and dendrite formation in *Drosophila*. Despite altered neuronal morphology, *Git* knockdown did not result in increased locomotor that has been observed for other *Drosophila* models of ADHD-associated genes (van der Voet et al., 2016). However, this may not be unexpected, as our results clearly show that the *GIT1* locus is not associated with ADHD risk, ADHD symptom counts, neuropsychological performance, or brain volume and white matter integrity variation in large human data sets. Although we demonstrated that genetic variation in the

GIT1 locus is not associated with ADHD in humans, we cannot rule out any other effects of the *GIT1* locus on different behavioral characteristics. The observed effect of *Git1* deficiency in mice on fear learning and adaptation to new environments (Won et al., 2011), may be interesting starting points for future studies in humans. Interestingly, a more recent study proposed that rare variants in *GIT1*, along with other genetic and environmental factors, cause dysregulation of PAK3 leading to synaptic deficits in schizophrenia (Kim et al., 2017b).

VII. What is the value of brain imaging genetics?

A possibility for combining neuroimaging and genetics is by exploring the effects of known disease genes on the brain. While association studies (e.g., GWAS) have identified genetic variants for ADHD, there is still a long way to go from genetic association to understanding the pathophysiological mechanism underlying its effect on ADHD risk. As shown in **Figure 3**, while genetic risk variants have in common that they confer risk for a particular disorder, the pathophysiological mechanisms by which they do so are not necessarily the same. Brain imaging genetics can shed light on which specific pathways are actually involved. Imaging genetics studies have been pursued with two main aims: First, such studies were suggested as a short-cut for the identification of ADHD candidate genes via the use of brain phenotypes (Bigos and Weinberger, 2010; Durston, 2010; Meyer-Lindenberg and Weinberger, 2006). As those brain phenotypes are thought to lie in-between a genetic factor and the clinical phenotype, it has been argued that effect size for effects of genes on those brain phenotypes may be larger than those for effects on behavior (Gottesman and Gould, 2003). However, for structural brain phenotypes, (i.e., brain volumes) this assumption has been refuted by recent large-scale studies (Franke et al., 2016). Overall, the success of this strategy (mainly candidate genes investigated) has been limited, as reviewed in **chapter 7** and **8**.

The second aim for using brain imaging genetics studies is to better understand the pathways leading from gene to disorder (via the brain and behavior). Most studies reviewed in **chapter 7** and **8** have used the design for this purpose. A nice example from literature for the successes achievable through this approach is the functional genetic variation in the *SLC6A4/5HTT* gene, for which converging evidence from different studies suggest that it is linked to emotion regulation through effects on brain activation in the amygdala and the wider 'threat circuit'. Studies of healthy participants and of different clinical cohorts have shown that those individuals carrying the risk factor for emotional dysregulation show increased activation in tasks related to emotion processing and learning (reviewed in **chapter 7**). Despite such promising results, our reviews of the existing literature in **chapters 7** and **8** show that existing work is severely flawed. Briefly, the main drawbacks are the small samples sizes of the individual studies (studies are underpowered), the fact that mostly only single genetic candidate variants/genes were investigated (hypothesis-driven studies), and that mainly region-of-interest analysis were performed (instead of brain-wide studies). Overall, only a very limited number of replication studies could be identified,

since most studies used different analysis strategies. For example, conflicting results with regard to the effects of schizophrenia risk variants on structural brain changes in the general population have been reported (Papiol et al., 2014; Van der Auwera et al., 2015). Obviously, alternative analysis approaches are highly needed. In **chapter 10** of this thesis, we revisited brain imaging genetics studies by performing a highly powered, genome-wide study. We investigated, whether the genetic contributions to ADHD risk and (subcortical) brain volume overlap. This was achieved by genome-wide analyses at two levels: first, at the level of the global genetic architecture, and, second, at the single variant level. On both levels, we found significant - though modest - genetic covariation between ADHD risk and brain volumes. Interestingly, genetic correlation between ADHD and ICV shows apparent specificity to this disorder, as it was not found in studies of other psychiatric disorders, e.g. schizophrenia (Adams et al., 2016b; Franke et al., 2016), major depressive disorder (Wigmore et al., 2016), or autism (Adams et al., 2016b). Overall, the degree of genetic overlap between ADHD and ICV was statistically modest. This may seem to be inconsistent with the general hypothesis that ADHD is a (or psychiatric disorders in general are) genetic-based brain disorder(s). However, several possible explanations for this modest amount of shared effects exist: All studies investigated brain structure at a gross anatomical measure and such atlas-based brain segmentations might be too coarse to identify subtler volumetric differences. Additionally, there is a limited link between structure and function at the level of MRI-derived volumes, so other neuroimaging phenotypes (structural and functional connectivity measures) could be more informative. With increasing availability of large consortia and/or biobank data sets, more hypothesis-generating studies will be performed.

Although the model of intermediate brain phenotypes can be helpful to provide a deeper understanding of the pathways from genes to the disorder, some caution is needed. Once stepped away from a clinical diagnosis, the results could become less (disorder-) specific. This means that the importance of shared neural substrates across psychopathologies need to be considered (Goodkind et al., 2015), as well as the high level of comorbidities in ADHD patients (e.g., anxiety disorders, autism spectrum disorders, major depressive disorder). For example, a reduction of (subcortical) brain volumes is not unique to an ADHD diagnosis, but is also seen in depression and bipolar disorder (Hibar et al., 2016; Schmaal et al., 2016). In addition, there is also evidence for substantial sharing of genetic risks across different psychiatric disorders. This extends to the level of characteristic traits throughout the population, with which some clinical disorders also share genetic risks (reviewed in (Martin et al., 2017)). The extent to which the shared genetic risk variants contribute to the shared neural substrates that are observed across psychiatric disorders, still needs to be determined.

Based on the results of the different studies reported in this thesis, it can be concluded that complementary methods are needed (1) to identify ADHD candidate genes (i.e., by a deliberate use of candidate gene studies and hypothesis-generating approaches and by looking at both common and rare variants) and (2) to validate the (functional) involvement

of such genes in ADHD (i.e., by combining statistical genetics approaches, bioinformatics, animal models, and brain imaging genetics). Moreover, replication studies and thorough validation of novel candidate genes are essential. Additionally, this thesis provides evidence for rare genetic variants playing a role in ADHD etiology, however their exact impact is still unknown.

Strengths and limitations

Approaches for gene identification

A main strength of this thesis is the integration of findings of many different samples, cohorts, and data sets. Whenever possible, information from large-scale international consortia (i.e. association summary statistics) was combined with data from well-phenotyped local and international cohorts (cohorts are summarized in **Table 1** in the **thesis introduction**). Although the maximization of sample size is always important for genetic association studies, smaller cohorts can be of importance as well, as they may be phenotyped more precisely (less measurement error and thus more homogeneous), and a broader phenotypic spectrum may be investigated. Essentially, this means that the trade-off between maximizing power by increasing sample size (thus potentially increasing heterogeneity) and more in-depth phenotyping in smaller (potentially less heterogeneous, but also more expensive per individual) cohorts needs to be considered.

A second strength is that the different chapters (which) of this thesis report a wide range of approaches for genetic association studies approaches. These reach from hypothesis-based analyses at candidate SNP level to hypothesis-generating genome-wide analyses to uncover different aspects of ADHD's genetic architecture. Whenever possible, we aimed at meta-analyzing data sets to maximize statistical power (**chapter 2** and **3**) or we used the largest data sets that were publicly available (GWAS meta-analysis summary statistics). Increase in statistical power was also achieved by aggregating association signals in a single statistical test instead of performing single variant association analyses only, as it was done by gene-based or gene-set association analyses (**chapter 3**, **4**, **5**, **6**, **9**, **10**). We also covered a broad spectrum of genetic variants. In **chapters 3**, **5**, and **6**, we investigated rare variants, other chapters focused on common genetic variants. In **chapter 5**, we specifically investigated the role of structural genetic variations (CNVs), in the other chapters single nucleotide variants (SNVs) were examined. A limitation of using association statistics data from large international consortia is that those often do not incorporate information on confounding factors, such as medication use or the presence of comorbid disorders. Additionally, all those data came from cross-sectional studies. However, longitudinal studies are essential if ADHD across the lifespan, including detailed information on remission and persistence, should be investigated. Moreover, most cohorts collected data only on a single

type of genetic variation for all individuals. However, it is known that each individual carries a combination of different types of genetic variants that jointly may cause ADHD.

Mapping mechanisms from gene to disorder

Chapter 7 and **8** contribute to the literature by providing a comprehensive literature review of brain imaging genetics studies for ADHD and additional neurodevelopmental disorders with high comorbidity (ASD and specific forms of ID). Given the inconsistency of the research findings, it is challenging for researchers to integrate findings and to provide robust conclusions based on existing studies. However, we uncovered important gaps in the current literature and pointed the reader to common pitfalls in imaging genetics studies. We formulated important directions for future brain imaging genetics studies. Integration of interdisciplinary research findings and the use of complementary approaches are essential to better map the molecular mechanisms involved in ADHD etiology. **Chapter 9** describes the results of such an attempt for a single gene, *GIT1*. There, we functionally characterized gene effects in *Drosophila*, by investigating both the morphology of neurons and ADHD-related behavioral phenotypes. Additionally, this chapter integrated results of genetic association analyses, brain imaging genetics, and mRNA expression analysis. We also addressed one of the limitations of imaging genetics studies by performing a genome-wide analysis (in **chapter 10**). With increasing availability of summary statistics derived from GWAS meta-analyses on brain imaging phenotypes, brain-wide approaches will contribute to improving the brain imaging genetics research field further. Future studies should also investigate interactions of the environment with genetic factors contributing to development of ADHD, as adverse prenatal and postnatal environmental exposures have been linked to both ADHD and general brain growth and development (Faraone et al., 2015).

(Clinical) implications

The underlying molecular and neurobiological mechanisms of ADHD are still not completely understood, and although pharmacological treatment options exist, these are limited especially in treating executive function deficits or disordered emotional regulation (Warikoo and Faraone, 2013). The drugs used to treat ADHD, only reduce symptoms, they are not curative. Additionally, not every patient with ADHD responds well to the existing treatment options. There is thus a need to better understand the underlying mechanisms leading to ADHD to enable the development of better medication. While this thesis describes fundamental research, it may contribute to the search for such novel treatment options (as well as potential diagnostic innovations and prevention) by increasing knowledge on ADHD etiology.

In the field of ADHD as well as in the whole of psychiatry, it is challenging to translate genome-wide association findings into diagnostic tools and new therapeutics for psychiatry (Breen et al., 2016). Currently, the genetic findings (and neuroimaging markers as well) are not suitable to diagnose psychiatric disorders, due to the overall limited amount of explained variance and the (resulting) lack of sensitivity and specificity. Examples from neurodegenerative disease show that the added predictive value of these genetic variants is low (Chouraki et al., 2016; Verhaaren et al., 2013). The challenges for the development of novel therapeutics for psychiatric disorders result from the paucity of novel, valid targets. This results from etiological heterogeneity, the complex and polygenic nature of genetic risk, and the definition of psychiatric disorders on the basis of the range and duration of symptoms (which can be subjective, self-reported, or observational) (Breen et al., 2016). Integration of genetic data downstream of genome-wide approaches may be used for target selection, but also for matching targets to indications; e.g., by allowing more accurate identification of high-risk individuals (Breen et al., 2016). Indeed, the evidence from genomics for a tractable biology underlying psychiatric disorders is promising, and the high degree of genetic pleiotropy suggests that therapeutic approaches may even be successful across current diagnostic boundaries (O'Donovan and Owen, 2016).

Although not directly a result of this thesis, it is important that clinicians increasingly take a developmental life-course approach ensuring that patients are effectively managed across the transition from childhood to and through adulthood. Developmental change over time should be expected and anticipated. Increased understanding of the factors contributing to the persistence of ADHD is essential in ultimately providing a better care for ADHD across the lifespan.

Important knowledge about ADHD etiology (e.g., obtained by interdisciplinary research) should efficiently be disseminated to the society in order to reduce the stigma of mental disorders. A central aspect therein is the concept of the genetic continuum, as it is discussed above. Given the fact that ADHD can be seen as an extreme of a continuum, treatment options or support may also be offered to individuals with subthreshold symptoms.

Challenges for the future

For genetic discoveries, the most obvious approach is to increase the *sample size* for discovery studies. This has been successful for other complex traits (Gormley et al., 2016; Okbay et al., 2016; Schizophrenia Working Group of the Psychiatric Genomics et al., 2014), and there is little doubt that this will also improve the power of genetic studies in ADHD and brain imaging phenotypes. Given the small effect sizes of common genetic variants, large study samples are needed. To be able to obtain these large data sets, (international) *collaborations*

have been created, such as IMpACT (Franke and Reif, 2013), the PGC (Schizophrenia Working Group of the Psychiatric Genomics et al., 2014), and iPSYCH (Pedersen et al., 2017), ENIGMA (Thompson et al., 2014), the Human Connectome Project (Van Essen et al., 2013), and the UK Biobank (Sudlow et al., 2015).

A second aspect is to maximize the information that can be extracted from already collected data (within data sets). For example, existing DNA material can be genome-wide genotyped or sequenced in order to increase density of genetic information. In current ADHD genetics research, basically two parallel research approaches have been considered: First, large consortia that localize the main differences between cases and controls. Secondly, deeply phenotyped (smaller but reasonably sized) data-collections at single sites. Such single site data sets are useful to work out the underlying mechanisms involved in different aspects of ADHD (e.g., quantitative behavior, brain, neuropsychology); these data will (potentially) be more homogeneous than a combined set of data sets. This may especially be important if different studies measure the same phenotypes, but use different instruments to assess these (e.g., adult ADHD symptoms as described in **chapter 3**). Additionally, large biobanks have been initiated in the past years (Elliott and Peakman, 2008; The German National Cohort, 2014), and the first batches of their data have already become available for analyses (Davies et al., 2016; Gale et al., 2016; Hagenaars et al., 2016; Lane et al., 2016; Lyall et al., 2016; Wain et al., 2015). Such growing resources in electronic health records and population/based registries linked to genomic data can now be used to investigate the pleiotropic effects of psychiatric risk variants across the phenome (Smoller, 2017). Biobanks are likely to receive a prominent role in genetic discoveries in the coming years.

An additional approach to boost statistical analyses for genetic discoveries is by using more *powerful statistical techniques*. This relates to the analysis of genetic variability as well as to the type/complexity of the phenotype that is being analyzed. Examples come from recent novel meta-analytical techniques that allow for combining results from multiple studies in a way that yields the same results as pooled analysis, but does not require the raw data to be shared, which allows for an increased sample size (Adams et al., 2016a). In addition, most studies focus on univariate linear regression models for analyzing associations between genetic variants and imaging markers or disease status. Here, there is room for improvement through more sophisticated (multivariate) methods including machine learning algorithms, e.g., deep learning, or support vector machines (Burges, 1998; LeCun et al., 2015). Future work will also likely focus on the integration of information from various sources of genetic variation at the level of the individual subject. In genetics, successful discovery for psychiatric disorders will increasingly depend on the ability to integrate genetic information from *de novo* and inherited, rare and common forms of variation in the genome. Accurate estimation of the genetic load across such variability will subsequently enhance our understanding of ADHD.

Additionally, statistical power can be improved in alternative ways, e.g., by *reducing the measurement error in both genetics and imaging*. For example, the haplotype reference consortium has pooled together 65,000 human haplotypes to create a reference panel to which genotypes with minor allele frequencies as low as 0.1% can still be reliably imputed (the Haplotype Reference, 2016). Furthermore, the use of common analysis protocols across collaborating sites can help to reduce between site heterogeneity, e.g. in behavioral phenotyping and in neuroimaging. Recent advances in DNA sequencing and reductions in the associated costs also pave the way for obtaining whole genome sequences (Davies, 2015). This may enable the identification of causal variants instead of tagging variants, in such instances in which the association signal is diluted and the linkage disquilibrium is not high enough. Similarly, imaging markers contain noise that is due to the equipment or subsequent image processing. Additionally, ultra-high field strength MRI scanners can provide more detail of the brain (Tallantyre et al., 2008; Tallantyre et al., 2010) and may be of great importance for future brain imaging genetics studies. Generally, imaging genetics studies should consider more consistent study designs. Confounding factors such as age, gender, symptom dimensions, comorbid disorders, and the genetic makeup should carefully be controlled for.

Most brain imaging genetics studies in ADHD up to now are cross-sectional studies. However, *longitudinal studies* are warranted to study developmental trajectories of brain changes in ADHD, as differences across the lifespan have been found in neuroimaging (Hoogman et al., 2017) and genetic results (Thissen et al., 2015), and provide a chance to study the potential role of such effects on persistence and remittance of ADHD. Especially longitudinal population-based cohorts with genomic data can help to discover trajectories of ADHD risk and gene-environment interplay (Riglin et al., 2016). Therefore it is critical that participants in biobanks and large-scale cohorts can be recalled, e.g., for further deep phenotyping. Another note on the *choice of study design* is about hypothesis-driven versus hypothesis-generating approaches. Hypothesis-generating approaches can provide new leads to follow. For example, genome-wide whole-brain analyses in ADHD, being hypothesis-generating on both genetic and neuroimaging data points, can generate new hypotheses (Stein et al., 2010). Although hypothesis-driven approaches have some limitations, investigation of candidate genes and candidate brain areas will remain useful in future research by providing insights into the underlying mechanisms in ADHD. However, candidates for these approaches should be selected downstream of the hypothesis-generating approaches in future studies.

The *selection of imaging markers* will have an important impact on future research in a more general sense. The structural measures that are frequently used so far are fairly gross measures and probably oversimplify the complexity of the structural volumes. Genes themselves might affect specific regions of the brain, and the use of an aggregate brain measure can make such localized effects difficult to find. Emerging imaging markers

that can be relevant for neuropsychiatric disorders, and specifically for ADHD, include (among others) the white matter microstructure. Future studies can extend the scope of imaging genetics to other clinically relevant and heritable imaging markers, including the microstructural integrity of white matter as measured by diffusion tensor imaging and functional connectivity assessed by functional MRI (Jahanshad et al., 2013; Meyer-Lindenberg, 2012). However, work by global collaborations is currently still hampered by the large heterogeneity of such data between different sites. One attempt to reduce such heterogeneity is the use of shared protocols for data acquisition and analyses.

Furthermore, *cross-investigations with other sources of biological data* (e.g., transcriptomics, proteomics, metabolomics, microbiomics) can amplify the synergistic value of imaging genetics. These data represent yet another dimension that can be added on top of imaging and genetics, and may therefore also require novel methods to be developed for facilitating such studies. Sophisticated integration of all types of 'omics' data will be helpful in turning the information about ADHD's genetic architecture into knowledge about its biological signature. Next to such interdisciplinary approaches, studies will also focus on proving causality. To dissect the functional effects of risk variants and to prove causality, mechanistic studies including cell or animal models are used. Statistical genetics approaches, such as mendelian randomization, also aim to contribute to our understanding of causal relationship between risk variants and risk for the disorder. Until now, such mendelian randomization approaches are mainly limited by lack of robust association findings, but this will probably be explored in more detail in the near future.

At least for psychiatric disorders, a substantial fraction of heritability is likely explained by interactions between genes and familial environment. This is seen in current estimates of SNP heritability, which are well below the heritability computed by twin studies. A further complication is that the environment likely contributes to pleiotropy. For psychiatric disorders we know of many shared environmental risks (e.g., anoxic episodes, exposures to toxins, low birth weight), each of which have small effects on disease risk (Faraone, 2017). Moreover, ADHD's association with reduced brain volumes may partly be driven by environmental effects, either independently or in interaction with genetic factors. Adverse prenatal and postnatal environmental exposures have been linked to both ADHD and general brain growth and development (Faraone et al., 2015). Consistently, monozygotic twins discordant for ADHD were found to have significant epigenetic differences among genes highly expressed in the developing cerebellum, striatum, and thalamus, in addition to genes functionally related to neurodevelopment and neurotransmitter regulation (Chen et al., 2017b). Therefore, future work will need to investigate *environmental influences*, e.g., by epigenetics studies, given the potential importance of DNA methylation variation in genes related to neurodevelopmental processes that play a key role in the maturation and stability of cortical circuits (Walton et al., 2017).

Key findings of this thesis

- None of the common *DRD5* alleles is associated with ADHD risk or ADHD symptom counts in adults (**chapter 2**).
- Common genetic variants within the *STXBP5-AS1* gene are associated with self-reported adult ADHD symptoms (**chapter 3**).
- Genes, known to carry rare mutations in ID, contribute to ADHD risk through common variants (**chapter 4**).
- In *Drosophila*, knockdown of *TRAPPC9* and *MEF2C* contributes to ADHD-related behavior through different pathways (**chapter 4**).
- Common genetic variants in *FBXO25* gene are associated with ADHD, and overexpression of the *FBXO25* orthologue in the fruit fly leads to increased locomotion (**chapter 5**).
- Combining linkage analysis and whole-exome sequencing in multi-generation pedigrees is a fruitful approach for identifying novel ADHD candidate genes (**chapter 6**).
- ADHD imaging genetics studies can provide insight into the link between genes, disease-related behavior, and the brain (**chapter 7** and **8**).
- In ADHD imaging genetics studies, single genetic variants were studied, mostly focussing on dopamine-related genes. While promising results have been reported, ADHD imaging genetics studies are hampered by methodological differences in study design and analysis methodology, as well as by limited sample sizes (**chapter 7** and **8**).
- Imaging genetics research is still in its early stages, and conclusions about shared mechanisms of neurodevelopmental disorders cannot yet be drawn (**chapter 7**).
- Future studies should invest into complementary approaches at multiple levels of biological complexity. By combining and integrating findings across levels, we may get a better understanding of biological pathways from gene to disease (**chapter 8**).
- Common genetic variants in *GIT1* are not associated with ADHD, and despite GIT1's regulation of neuronal morphology, alterations in gene expression do not appear to have ADHD-related behavioral consequences (**chapter 9**).
- ADHD and intracranial volume are significantly negatively correlated. Individual loci of interest point to an involvement of neurite outgrowth-related genes (**chapter 10**).

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APPENDIX

Nederlandse samenvatting, acknowledgements, curriculum vitae, list of publications and Donders Graduate School for Cognitive Neuroscience

Nederlandse samenvatting

Attention-Deficit/Hyperactivity Disorder (ADHD), ook wel aandachtstekort-hyperactiviteitstoornis genoemd, is een veelvoorkomende ontwikkelingstoornis. Het wordt gekenmerkt door aandachtsproblemen, overmatige activiteit of moeite met het controleren van gedrag dat niet geschikt is voor iemands leeftijd. De symptomen verschijnen meestal voordat een persoon twaalf jaar oud is en voor een psychiatrische diagnose moeten de symptomen langer dan zes maanden aanwezig zijn en problemen veroorzaken in ten minste twee instellingen (zoals school, thuis of recreatieve activiteiten). Bij kinderen kunnen aandachtsproblemen leiden tot slechte schoolprestaties. Ondanks dat het een van de meest voorkomende en gediagnosticeerde psychische stoornissen is bij kinderen en adolescenten, is de exacte oorzaak in de meeste gevallen vrijwel onbekend. We weten uit eerder onderzoek dat genetische factoren een rol spelen. Daarnaast zijn er op groepsniveau verschillen gevonden in de bouw en het functioneren van de hersenen tussen mensen met en zonder ADHD.

Het doel van de studies beschreven in dit proefschrift was om genetische mechanismen te identificeren die betrokken zijn bij deze hoogst erfelijke aandoening ADHD en inzicht te krijgen in de onderliggende biologische mechanismen. Deel 1 van dit proefschrift (**hoofdstuk 2** t/m **6**) richt zich op de identificatie van genetische mechanismen die betrokken zijn bij ADHD. Hiervoor werd gebruik gemaakt van verschillende methoden uit de moleculaire en statistische genetica. In deel 2 van dit proefschrift (**hoofdstuk 7** t/m **10**) ligt de nadruk op het combineren van hersenmaten en genetica om verbanden te leggen tussen genetische risicofactoren en veranderingen in de hersenen. Hieronder wordt kort beschreven wat er in de individuele hoofdstukken werd onderzocht en gevonden.

Hoofdstuk 2 beschrijft een kandidaat gen studie, waarin de associatie van een polymorfisme van het *DRD5* gen met ADHD bij volwassenen werd onderzocht. Hiervoor werd een tot nu toe grootste meta-analyse uitgevoerd en werd de associatie van de *DRD5*-allelen met categorisch gedefinieerde ADHD en met onoplettende en hyperactief-impulsieve symptomen getest. In tegenstelling tot eerdere rapporten over ADHD bij kinderen, is geen van de *DRD5*-allelen geassocieerd met ADHD of met ADHD symptomen bij volwassenen.

In **hoofdstuk 3** hebben we een hypothesegenererende benadering toegepast en genetische varianten op genoomwijde schaal onderzocht. In dit hoofdstuk worden de resultaten gerapporteerd van een genoomwijde associatie meta-analyse van zelfgerapporteerde ADHD symptomen bij volwassenen. Genetische varianten binnen het *STXBP5-AS1* gen vertoonden de sterkste associatie, die werd gevalideerd in een meta-analyse van ADHD symptoomscores uit de kindertijd. Met behulp van een *in vitro* celassay werd aangetoond dat expressie van *STXBP5-AS1* de expressie van *STXBP5* verhoogde. Van STXBP5 is bekend dat het betrokken is bij de SNARE complexvorming, een belangrijk proces bij het transport van neurotransmitters in zenuwcellen. Bovendien correleerde

de expressie van *Stxbp5-AS1* in de prefrontale cortex met motorische impulsiviteit in verschillende muizenlijnen. Deze resultaten suggereren dat het *STXBP5-AS1* gen betrokken is bij het ontstaan van ADHD symptomen en wijzen op een rol van vesikeltransport van neurotransmitters als een biologisch mechanisme voor ADHD-gerelateerde impulsiviteitniveaus.

In **hoofdstuk 4** hebben we onderzocht of genen, waarvan bekend is dat als ze zeldzame mutaties dragen verstandelijke beperkingen veroorzaken, ook via vaker voorkomende genetische varianten bijdragen aan een verhoogt risico op ADHD. Hiermee wilden we nieuwe risicogenen voor ADHD identificeren. Een set van 396 genen, die eerder geassocieerd waren met verstandelijke beperkingen, was significant geassocieerd met ADHD in twee onafhankelijke cohorten. Genetische varianten in drie genen, *MEF2C*, *TRAPPC9* en *ST3GAL3*, werden geïdentificeerd als consistente topassociatie bevindingen. Vervolgens werden de nieuw geïdentificeerde ADHD kandidaat-genen functioneel gevalideerd met behulp van de fruitvlieg *Drosophila melanogaster*. Deze studie bevestigde de genetische overlapping van ADHD en verstandelijke beperkingen en we hebben verschillende nieuwe risicogenen voor ADHD geïdentificeerd, gevalideerd en functioneel gekarakteriseerd.

In **hoofdstuk 5** werd een stapsgewijze strategie gebruikt om een nieuw ADHD risicogen te identificeren en te valideren, waarbij de kracht van een familieonderzoek werd gecombineerd met de kracht van een grootschalige genoomwijde associatie studie. In een enkele familie, ernstig aangedaan door ADHD en comorbide psychiatrische stoornissen, ontdekten we een structurele genetische variant, die drie eiwitcoderende genen (*ZNF596*, *FBXO25* en *TDRP*) bevat, en deze segregeerde met ADHD in de familie. Deze geïdentificeerde nieuwe ADHD kandidaat-genen werden getest - individueel en gecombineerd - op tekenen van associatie met ADHD en andere relevante psychiatrische stoornissen. Vergeleken met de genetische achtergrondcontrole leidde verhoogde expressie van de *FBXO25*-ortholoog in *Drosophila melanogaster* tot verhoogde locomotorische activiteit. Deze studie wijst op een rol voor het *FBXO25* gen bij het ontstaan van ADHD.

In **hoofdstuk 6** wordt een innovatieve aanpak beschreven die linkage-analyse en whole-exome sequencing (WES) combineert in ADHD families met meerdere generaties. In totaal werden 24 genen geselecteerd die door WES-geïdentificeerde zeldzame genetische varianten bevatten. Die genen werden gezamenlijk geanalyseerd in gen-set analyses met behulp van gegevens van een grootschalig onafhankelijk exome-chip cohort van patiënten met persisterende vorm van ADHD en controles. Gen-wijde analyse voor het *AAED1*-gen bereikte significantie en een enkele variant binnen *AAED1* segregeerde met ADHD in de familie.

In **hoofdstuk 7** werd het effect van genetische risicofactoren op de structuur en de functie van de hersenen onderzocht. We gingen uit van bekende genetische risicofactoren voor een groep van aandoeningen die regelmatig samen voorkomen: ADHD, autisme spectrum stoornis, en bepaalde vormen van verstandelijke beperkingen. We onderzochten

per aandoening welke mechanismen in het brein worden beïnvloed door de genetische risicofactoren en probeerden mechanismen te vinden die geassocieerd zijn met de klinische overlap tussen de aandoeningen. We vonden dat genetische variatie in het serotonine transporter gen (*SLC6A4*/*5HTT*) consistent geassocieerd is met verhoogde activiteit van de amygdala en gerelateerde hersengebieden, in combinatie met een verminderde connectiviteit tussen deze gebieden, allemaal gerelateerd aan emotieregulatie. Op basis van de onderzochte literatuur, konden we specifieke aanbevelingen voor toekomstig onderzoek geven. Er is een grote noodzaak voor grotere studies, die hypothesevrij naar alle hersengebieden kijken, en die meer genen onderzoeken. Daarnaast is het nodig om te onderzoeken of veranderingen in de hersenen de effecten van genetische risicofactoren op het klinische fenotype kunnen verklaren. Naast het geven van een uitgebreid overzicht van de literatuur toonde dit onderzoek aan dat het combineren van genetica en hersenmaten nuttig is om meer te weten te komen over verbanden tussen genen, hersenen, en gedrag. Toch is het werkveld nog in een vroeg stadium en kunnen er nog geen definitieve conclusies getrokken worden over de overlap tussen de verschillende onderzochte aandoeningen.

In **hoofdstuk 8** hebben we ons werk uit het vorige hoofdstuk uitgebreid en een systematische literatuuronderzoek uitgevoerd, waarin we het effect van specifieke genetische risicofactoren van 62 ADHD kandidaat-genen op de structuur en functie van de hersenen onderzochten. Vrijwel uitsluitend werden individuele genetische varianten bestudeerd, voornamelijk van dopamine-gerelateerde genen. De conclusie van dit literatuuronderzoek was dat, hoewel veelbelovende resultaten zijn gemeld, 'brain imaging genetics' onderzoeken naar ADHD tot nu toe worden belemmerd door methodologische verschillen in onderzoeksopzet en analysemethodologie, evenals beperkte steekproefgroottes. In dit hoofdstuk bespreken we ook uitgebreid de behoefte aan complementaire methoden voor de evaluatie van de mechanismen die ten grondslag liggen aan ADHD risicogenen. Verder werd het belang van het combineren en integreren van bevindingen op verschillende niveaus benadrukt om daardoor een beter begrip over de biologische mechanismen van gen tot stoornis te verkrijgen.

In **hoofdstuk 9** wordt een integrale benadering beschreven die gericht was op het repliceren van een eerder gerapporteerde associatie van het *GIT1* gen met ADHD en op het onderzoeken van de rol ervan in cognitieve en hersenfenotypen. Associatie van *GIT1* met ADHD of gerelateerde fenotypes, zoals aanhoudende aandacht, werkgeheugen en hersenvolumematen werden niet bevestigd in veel grotere steekproefomvang dan in het primaire onderzoek. Wel werd aangetoond dat een functionele genetische variant de expressie van *GIT1* beïnvloed. Bovendien veroorzaakte *Git* knockdown in *Drosophila* abnormale synaps- en dendrietmorfologie, maar had geen invloed op het gedrag (dat wil zeggen de locomotorische activiteit). Onze resultaten geven aan dat, ondanks GIT1's regulatie van de neuronale morfologie, veranderingen in genexpressie geen effect op

ADHD-gerelateerd gedrag lijken te hebben en het hersenvolume bij mensen niet lijken te veranderen.

In **hoofdstuk 10** werd de genetische overlap van ADHD met subcorticale hersenvolumes en intracranieel volume (ICV) onderzocht. In overeenstemming met de fenotypische observatie werd een significante negatieve genetische correlatie tussen ADHD en ICV ontdekt, wat betekent dat een verhoogd risico op ADHD gecorreleerd is met een verminderde ICV. Meta-analyse van individuele varianten toonde aan dat significante genomische loci waren geassocieerd met zowel ADHD-risico als ICV; extra loci werden geïdentificeerd voor ADHD- en amygdala-, caudate nucleus- en putamenvolumes. Deze bevindingen, samen met resultaten van exploratieve gen-wijde en gen-set analyses suggereren dat hierbij genen een rol spelen die betrokken zijn bij de uitgroei van neuriten.

Door gebruik te maken van uiteenlopende analysemethoden, geven de studies beschreven in dit proefschrift nieuwe inzichten in de genetische architectuur en individuele genetische risicofactoren die bijdragen aan ADHD. Bovendien helpen deze studies om het biologische mechanisme van gen tot stoornis beter te schetsen door gebruik te maken van diermodellen en het combineren van genetische informatie met hersenmechanismen die betrokken zijn bij ADHD.

Acknowledgements

I wish to thank my supervisors for their patience and accuracy with which they made sure everything went its right way, for their valuable suggestions to the planning of research projects and for the intelligent editing of the manuscripts. Especially Barbara, who takes no idea, no comma unchecked. She has always been an enormous source of inspiration and motivation for me. Her knowledge, energy, discipline and enthusiasm for all research areas and science in general are admirable. She has this great ability to find the right balance between offering guidance and allowing me to explore tracks independently. I am extremely grateful for her trust, all the freedom and the endless opportunities she created for me to learn and to develop. Evenly important, she introduced me to her huge international network of which I profit a lot. This created various opportunities for me to travel to conferences, allowing me to present my work and to meet other scientists. Our work is only possible if we join forces and work together in large, international collaborations and one of the driving forces behind those efforts is Barbara – thank you. Besides working together on these international platforms – which I think is the actual beauty of our job – this also allowed me to make friends all over the world. Especially in today's political times, this can be considered as being extremely important.

Particularly significant impact also had Alejandro, who always knew how to challenge my thoughts and ideas. I really appreciate his critical mind and can only thank him for his unconditional faith in me as a researcher. He always welcomed a scientifically-sound discussion and argumentation, but also valued my position in case I had a different opinion. I wish to thank him for his honest interest in my personal and work-related well-being and I am grateful for feeling understood and being supported in such a liberal way.

Also worth mentioning is David, who has been more silently present in the background. Thanks to his eagles eyes', while carefully reading the manuscript, the last spelling mistakes were taken out.

While working on this project, I was fortunate enough to be able to discuss my thoughts for many hours with knowledgeable and patient friends and colleagues. Of particular note are Janita, my former PhD-student buddy and now co-promoter (☺), whose thoughts and suggestions are a constant source of inspiration; Nina, without whose advice the completion of this project would have been way more challenging (with whom should I have (endlessly) discussed all work- and life-related topics then?); and Martine, whose knowledge and experience in the field of ADHD, neuropsychology, brain imaging and general research practicalities were of great value for me.

I also wish to thank the colleagues of the Department of Human Genetics at the Radboudumc, especially all colleagues of the research lab of Multifactorial Diseases - for what I have learned from them over the years, for the climate they have created and which is a unique environment for studying ADHD brain imaging genetics. This lab is simply great to work with. It is amazing how enormously helpful everybody is and I wish to thank them all for their great company during the last years and for all their support through ups and downs of my PhD project.

Over the last years I had the great honor to collaborate with an immense number of people from the Donders Institute for Brain, Cognition and Behaviour, the Department of Human Genetics, the Department of Psychiatry, the Department of Health Evidence and the Max-Planck Institute. I can only thank them all for their valuable collaborations, I am looking forward to continue our collaborations.

Also outside of Nijmegen I had the opportunity to be involved in the work of various international consortia, such as IMpACT, ENIGMA, PGC, iPSYCH and Aggressotype. Without these global efforts and very fruitful collaborations, this PhD project would not have been as successful as it was now.

My friends and family. For everything.

Curriculum Vitae

Marieke Klein obtained a Bachelor's degree in Biology in 2010, followed by a Master's degree in Medical Biology in 2012 at the Radboud University in Nijmegen. During her final Master's internship at the Leibniz Institute for Neurobiology, Magdeburg, Germany, she examined the effects of genetic variants on learning and memory in human subjects by using functional MRI. She continued this line of research by combining genetics and brain imaging in her PhD project. From 2013 onwards, she worked as a PhD student at the department of Human Genetics at the Radboudumc and the Donders Institute for Brain, Cognition, and Behaviour. Her work focused on the complex genetics of Attention-Deficit/Hyperactivity Disorder (ADHD) and ADHD imaging genetics conducted within the IMpACT consortium, which aims at identifying novel genetic variants for adult ADHD and at understanding the mechanisms underlying the effect of these genetic variants on disease risk. The results of this project are described in this thesis and were also presented at several international conferences, including oral presentations at the Annual Meeting of the Society of Biological Psychiatry in 2017, the Eunethydis international conference meetings in 2016 an 2018, and the World Congress of Psychiatric Genetics in 2018. In 2016, she visited the lab of Benjamin M. Neale at the Broad Institute/Stanley Centre for Psychiatric Research in Boston, USA, to learn state-of-the-art statistical genetics methods in order to identify shared genetic overlap between ADHD and structural brain measures. Additionally, she was involved in the daily supervision of several Bachelor and Master students and organization and teaching of various Bachelor and Master programmes at the Radboudumc and the Radboud University. Marieke continues her work as a post-doctoral researcher at the department of Human Genetics of the Radboudumc in Nijmegen and at the department of Psychiatry of the University Medical Center in Utrecht. Here she investigates the effects of common genetic factors on aggressive and anti-social behavior and their contribution to brain development.

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